A Peptide from the Heptad Repeat of Human Immunodeficiency Virus gp41 Shows both Membrane Binding and Coiled-Coil Formation†

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ABSTRACT: The envelope glycoprotein gp41 from human immunodeficiency virus type 1 (HIV-1) is involved in membrane fusion and virus entry. It contains a functionally important leucine zipper-like heptad repeat region (residues 553−590). To investigate the solution structure and membrane-binding properties of this region, cysteine-substituted variants of a 38-residue peptide derived from the heptad repeat were synthesized and modified with nitroxide spin labels. Analytical equilibrium ultracentrifugation studies indicated it is primarily tetrameric in solution, in contrast to the protein gp160 which is a mixture of trimers and tetramers. Electron paramagnetic resonance (EPR) measurements indicated that the peptide was bound to vesicles containing 10 mol % negatively charged lipids. The peptides were bound parallel to the membrane surface, near the water−membrane interface, in a structure different from the solution structure, most likely as monomers. When Asp, Pro, or Ser was substituted for Ile at the core "a" position of the heptad repeat in the middle of the peptide, the coiled coil was destabilized. In addition, these peptides showed reduced membrane-binding affinities. Thus, mutations that destabilized coiled-coil formation also decreased membrane-binding propensity. These experimental results, taken with previous evidence, suggest two functions for the heptad repeat of gp41 after CD4 binding: (1) to form an extended coiled coil; (2) to provide a hydrophobic face that binds to the host-cell membrane, bringing the viral and cellular membranes closer and facilitating fusion.

Retrovirus surfaces are coated with envelope (*env*) glycoproteins. These proteins are synthesized as a single-polypeptide precursor that is subsequently cleaved into a surface attachment subunit (SU)[1] responsible for cell recognition and a fusion peptide containing a transmembrane domain (TM) responsible for viral-cell membrane fusion. After cleavage, the two domains are still attached. Specifically, for HIV-1, the *env* protein gp160 is cleaved into CD4 receptor-binding gp120 (SU) and gp41 (TM). They are noncovalently associated (Allan *et al.*, 1985; Robey *et al.*, 1985; Veronese *et al.*, 1985, McCune *et al.*, 1988). This is analogous to the most-studied *env* protein, influenza virus hemagglutinin (HA), which is cleaved into the sialic acid binding HA1 (SU) domain and the host-cell membrane interacting HA2 (TM) domain. These two domains are linked by a disulfide bond. The HA complex is trimeric (Wilson *et al.*, 1981). In contrast, the mature gp120/gp41 complex is a mixture of tetramers and trimers (Pinter *et al.*, 1989; Earl *et al.*, 1990).

Recent progress on HA provides many clues to the function of TM domains. The influenza virus fuses with the host cell in endosomes when the pH is lowered. Carr and Kim (1993) identified a sequence in HA2 with a leucine zipper-like heptad repeat and high helical propensity that is actually a loop in the neutral pH structure. Furthermore, they found that a peptide from this region is unstructured at neutral pH but becomes a three-stranded coiled coil at endosomal pH. Comparison of low- and neutral-pH crystal structures of the HA2 domain confirmed that this region becomes a coiled coil upon acidification and exposes the fusion peptide at the top of the ectodomain (Bullough *et al.*, 1994). Furthermore, Yu *et al.* (1994) found that this peptide binds to lipid bilayers under similar conditions, suggesting membrane fusion is further facilitated by the insertion of this region into the target membrane.

The HIV-1 protein gp41 has an analogous region, depicted in Figure 1, which is predicted to be a coiled coil by computer algorithms (Gallaher *et al.*, 1989; Delwart, 1990). A 38-residue peptide derived from this region is in fact 85% helical at 10 μM and 37 °C (Wild *et al.*, 1992). The thermal stability of the helix is concentration dependent, indicating it is oligomeric. Furthermore, this peptide blocks virus-mediated cell−cell fusion in HIV-infected cells at 5 μg/mL. A disulfide-linked dimer lowers the necessary concentration to 1 μg/mL, suggesting multimerization is involved. It has been suggested that the heptad repeat region is responsible for oligomerization (Gallaher *et al.*, 1989; Delwart *et al.*, 1990). However, mutations in gp160 at the hydrophobic core-forming "a" position of the heptad repeat expected to destabilize the coiled coil affected neither oligomerization nor insertion of gp160 into the viral membrane, suggesting the protein is properly folded in the prefusogenic state (Dubay *et al.*, 1992; Chen *et al.*, 1993). Although oligomerization was not affected, these mutations did impair virus

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; EPR, electron paramagnetic resonance; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; SU, surface attachment subunit; TM, transmembrane subunit; HA, influenza virus hemagglutinin; HPLC, high-performance liquid chromatography; MOPS, 3-morpholinopropanesulfonic acid; hr, heptad repeat; wt, wild type; CD, circular dichroism; Gu·HCl, guanidine hydrochloride; τ_R, rotational correlation time.

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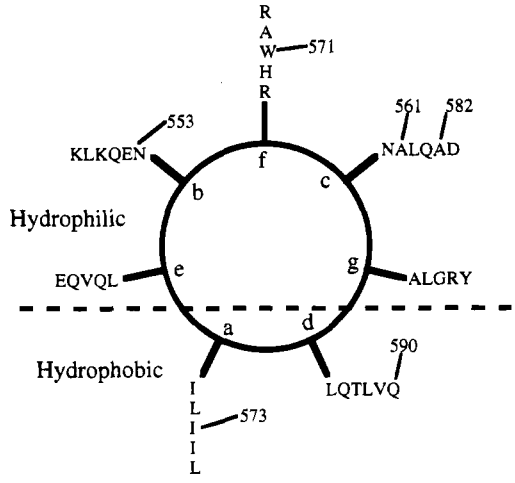

FIGURE 1: The heptad repeat region of HIV gp41 (residues 553–590 of the HIV-1_{HXB2R} isolate) displayed as a helical wheel.

infectivity, implying that this region of gp41 is crucial for some other aspect of the virus life cycle, such as membrane fusion and virus entry. It has been suggested that gp120 binding to CD4 receptors on the host cell triggers the heptad repeat region to change from a loop to an extended coiled coil, which exposes the fusion peptide to the host-cell membrane (Dubay *et al.*, 1992; Chen *et al.*, 1993; Wild *et al.*, 1994).

Here we study the oligomerization in solution and the membrane-binding behavior of a 38-residue peptide derived from the heptad repeat region of gp41. The amino acid sequence of the peptide is the same as that studied by Wild *et al.* (1992, 1994), except for spin-label modification to allow study by EPR. It was found that this peptide forms a tetrameric coiled coil in solution. It also binds to negatively charged phospholipid vesicles parallel to the membrane surface, presumably as a monomer. Mutations analogous to those made by Dubay *et al.* (1992) reduced both coiled-coil formation and membrane binding. These results support the hypothesis that after coiled-coil formation this region dissociates, exposing the hydrophobic interior of the coiled coil. This hydrophobic face subsequently binds to the host-cell membrane, promoting fusion by bringing the two membranes closer together.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) were obtained from Avanti Polar Lipids (Birmingham, AL). 3-(2-Iodoacetamido)-PROXYL was obtained from Sigma (St. Louis, MO).

Peptide Synthesis. The peptide corresponding to residues 553–590 of gp41, cysteine-substituted variants, and other variants were synthesized by solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) amino acids (Fields & Noble, 1990) and Rink amide resin from Nova Biochem (La Jolla, CA). All peptides were acetylated at the amino terminus with acetic anhydride. Following synthesis, peptides were cleaved from the resin and protecting groups were removed by treatment with a solution containing 85.5% trifluoroacetic acid, 5% water, 4.5% phenol, 2.5% anisol, and 2.5% dithioethanol for 5 h at room temperature (King *et al.*, 1990), and then the peptides were precipitated in *tert*-butyl methyl ether. A Vydac C18 reversed-phase high-performance liquid chromatography (HPLC) column was used to purify the peptides using a 35%–65% water/acetonitrile gradient containing 0.1% trifluoroacetic acid. The identities and purities of the peptides were confirmed by electrospray-ionization mass spectrometry.

Spin Labeling. Cysteine-substituted variants were spin labeled with thiol-specific 3-(2-iodoacetamido)-PROXYL. A 3-fold molar excess spin label in dimethyl sulfoxide was added to peptides dissolved in 10 mM MOPS buffer, pH 7.0. After being reacted for 1 h at room temperature, the peptide was purified with HPLC. The unreacted spin label was eluted much earlier than the spin-labeled peptide, simplifying the purification. The single peak containing the spin-labeled peptide was isolated and lyophilized. The identities and purities of the spin-labeled peptides were again confirmed by electrospray-ionization mass spectrometry.

Vesicle Preparation. POPC and POPG were dissolved in chloroform and mixed to obtain the desired ratio of negatively charged lipid. The solvent was removed by a stream of nitrogen, and trace amounts of chloroform were removed by placing the lipid mixture in high vacuum for at least 1 h. The dry lipid was then resuspended in 20 mM MOPS buffer, pH 7.2, yielding a final lipid concentration of 65 mM. Vesicles were prepared by passing the mixture through 100 nm pore size polycarbonate membranes using a mini LiposoFast extruder (Avestine, Canada) after five cycles of freeze–thaw. When vesicles were mixed with peptide solutions, the final lipid concentration was 48 mM and the peptide concentration was 50 μM.

Electron Paramagnetic Resonance (EPR) Spectroscopy. EPR measurements were performed at 22 °C using a Bruker ESP300 EPR spectrometer (Bruker, Germany) equipped with a low-noise microwave amplifier (Miteq, Hauppauge, NY) and a loop–gap resonator (Medical Advances, Milwaukee, WI). The modulation amplitude was set at no greater than one-fifth of the line width.

Circular Dichroism (CD) Spectroscopy. CD spectra were measured at 22 °C in 20 mM potassium phosphate, pH 7.2, and 10 μM peptide using a Jasco J600 CD spectrometer and a 1.0 cm pathlength cell. Peptide concentrations were determined from either A_{280} or $A_{275.5}$ (Edlehoch, 1967).

Analytical Ultracentrifugation. Sedimentation equilibria were determined with a Beckman Optima XL-A ultracentrifuge. The speed was 40 000 rpm, and the radius was from 5.80 to 7.20 cm. The peptides were in a buffer of 19.45 mM sodium phosphate and 0.275 mM citric acid at pH 8.0 and 4 °C. The solvent density was calculated to be 1.008 g mL^{-1} from standard tables (Weast, 1975), and the partial specific volume of the gp41 heptad repeat peptide with wild-type sequence ("hr.wt") was calculated to be 0.7616 cm^3 g^{-1} (McMeekin *et al.*, 1949).

RESULTS

To investigate coiled-coil formation and membrane binding of the peptide derived from the leucine zipper-like heptad repeat region of gp41, we synthesized seven peptide variants (Table 1). The peptide hr.wt (heptad repeat, wild type) corresponds to residues 553–590 from the heptad repeat region of HIV-1_{HXB2} gp41 and is the same as the peptide dp107 studied by Wild *et al.* (1992). The spin label 3-(2-iodoacetamido)-PROXYL was attached at the cysteine residues of all the peptides except hr.wt. The peptides fall into two classes: those with conservative mutations and those

Table 1: Peptides[a]

hr.wt	NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ
hr.n	.....C.....
hr.m	.....C.....
hr.c	.....C.....
hr.D	.....C.D.....
hr.S	.....C.S.....
hr.P	.....C.P.....

[a] Residues 553−590 from the heptad repeat region of HIV-1_{HXB2} gp41. Cys was spin labeled.

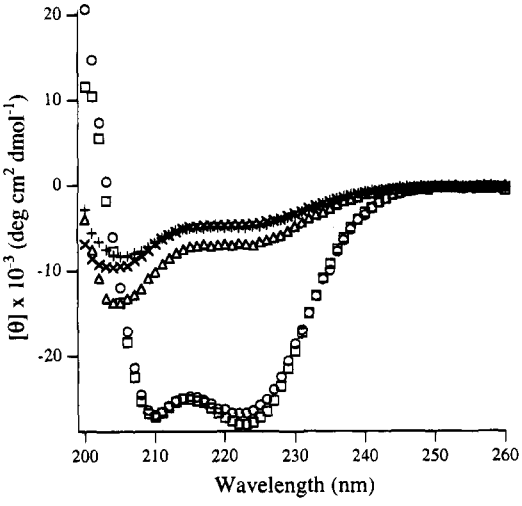

FIGURE 2: CD spectra of hr.wt (□) and the spin-labeled peptides hr.D (+), hr.P (×), hr.S (△), and hr.m (○) at 22 °C and 10 μM. The spectra of hr.c and hr.n were nearly identical to that of hr.m.

with disruptive mutations expected to greatly perturb coiled-coil formation. The peptides hr.n, hr.m, and hr.c correspond to the wild-type peptide, except that certain residues are changed to cysteine to allow attachment of cysteine-specific nitroxide spin labels near the amino terminus (hr.n), in the middle (hr.m), or near the carboxyl terminus (hr.c) of the peptide. The substitution site is at the "f" position for hr.m (W571C) and at the "c" position for both hr.n and hr.c (A561C and A582C, respectively). Mutations at the solvent-exposed f and c positions are not anticipated to affect coiled-coil formation (Hodges *et al.*, 1972; Parry, 1982; Harbury *et al.*, 1993). This was supported by the observation that CD spectroscopy, which is sensitive to secondary structure, shows little difference between spin-labeled peptides and the wild-type peptide (Figure 2). These spectra are consistent with those previously observed (Wild *et al.*, 1992).

If the hr peptides containing conservative mutations are oligomeric, then the distance between nitroxide spin labels will be less than 18 Å. This is close enough for the unpaired electrons to interact via spin-exchange interactions, thereby broadening the EPR spectrum (Luckhurst, 1976; Miick *et al.*, 1992; Fiori *et al.*, 1993). However, adding excess unlabeled peptide to oligomeric labeled peptides should result in heterooligomers containing only one spin label per oligomer, eliminating spin-exchange interactions and narrowing the EPR spectrum. Figure 3 shows that, when

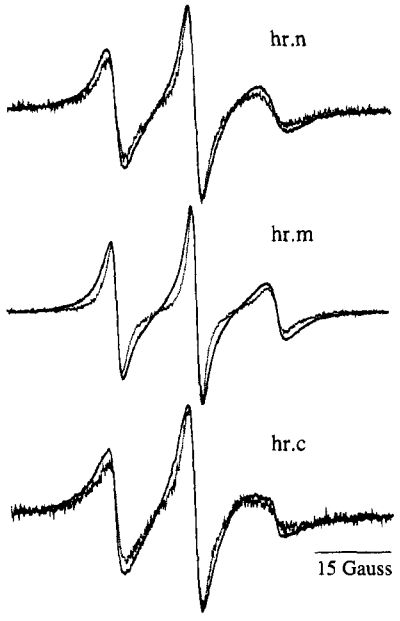

FIGURE 3: EPR spectra of 100 μM hr.n, hr.m, and hr.c in 20 mM MOPS, pH 7.2 (solid line), and of 50 μM hr.n, hr.m, and hr.c with 300 μM hr.wt (dashed line). The peptides hr.n and hr.c are labeled at the "c" position, while hr.m is labeled at the "f" position. The f position is more solvent exposed and thus has more mobility, as seen in the sharper EPR spectrum.

unlabeled wild-type peptide was added to labeled hr.m, hr.n, and hr.c, the EPR line width narrowed, as expected for heterooligomers. EPR spectra of these peptides mixed with hr.wt show a rotational correlation time (τ_R) in the range 1−2 ns (Schneider & Freed, 1989), characteristic of peptides with nonrandom coil structure. If the peptides were monomers, adding unlabeled peptide would have no effect on the EPR line shape. Thus, the EPR spectra show that the peptides are oligomeric.

CD data indicate 10 μM hr.wt is approximately 85% helical at 37 °C (Wild *et al.*, 1992). However, this is a measure of total helicity and not necessarily oligomerization. The peptides could be 100% oligomerized but with frayed ends (Fiori *et al.*, 1993). Likewise, there could be an equilibrium between unstructured monomers and completely helical oligomers. EPR spectra are sensitive to the rotational correlation times of the nitroxide spin label. Random coil peptides are more disordered than coiled coils, and thus lead to a faster rotational correlation time of the nitroxide and a sharper spectrum (Schneider & Freed, 1989). As the peptide concentration was decreased, a sharp component in the spectrum increased, indicating the fraction of random coil monomer is higher at low concentration (Figure 4). Thus, there is a monomer−oligomer equilibrium. If the peptide were 100% oligomerized but had frayed ends, resulting in 85% helicity, then the EPR spectrum would not vary significantly with concentration.

Knowing that conservative hr peptides form a mixture of monomers and oligomers, we used analytical equilibrium ultracentrifugation to determine the oligomerization state of hr.wt in solution. A two-component model consisting of only solvent and a single molecular weight species fit none of the data, consistent with the concentration-dependent EPR study. Because EPR data show evidence of disordered monomeric peptide in equilibrium with ordered oligomers (Figure 4), we then compared the ultracentrifugation data to

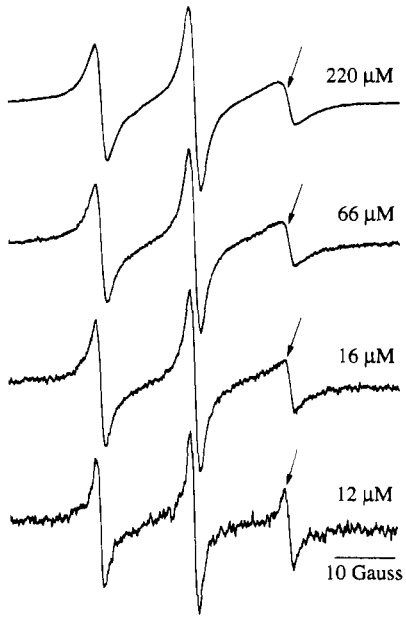

FIGURE 4: EPR spectrum of hr.m at different concentrations. The arrow identifies the unstructured monomeric component, which has a sharper spectrum due to faster motion.

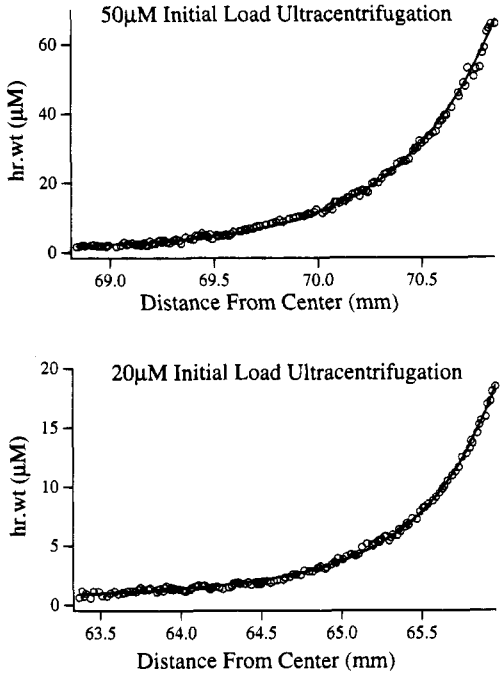

FIGURE 5: Ultracentrifugation data from 50 and 20 μM initial load concentration of hr.wt (open circles) and the best fit (solid lines) as described in the text.

a model consisting of monomers in equilibrium with oligomers of unknown molecular weight. The molecular weight was varied to obtain a best fit to the data using the Levenberg–Marquardt algorithm. The data for an initial loading concentration of 50 μM fit best to monomers and 18.1 kDa oligomers, which is the calculated molecular weight of a tetramer (Figure 5). Data from an initial loading concentration of 20 μM best fit monomers and 19.6 kDa oligomers, which also suggests the peptide forms tetramers, in equilibrium with monomers (Figure 5). This is in agreement with a recent study which indicates that when a fusion protein consisting of the heptad repeat region of gp41 and part of bacterial protein A is made, the fusion protein forms multimers higher order than dimeric (Bernstein *et al.*, 1995). From these data a tetramer dissociation constant of $(2.5 \pm 1) \times 10^{-18}$ M^3 was calculated.

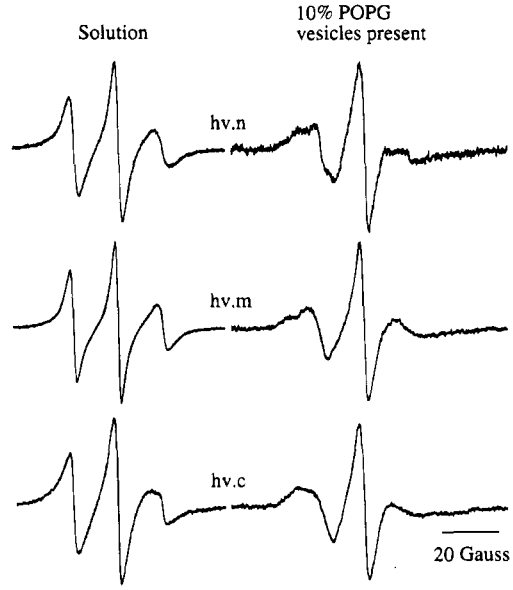

FIGURE 6: EPR spectra of 100 μM hr.n, hr.m, and hr.c in 20 mM MOPS, pH 7.2, and of 50 μM hr.n, hr.m, and hr.c in 20 mM MOPS, pH 7.2, in the presence of lipid vesicles composed of 90 mol % POPC and 10 mol % POPG. The total lipid concentration was 48 mM.

Furthermore, a dissociation constant from 3.0×10^{-18} to 1.5×10^{-17} M^3 was calculated from the EPR data of 12 μM hr.m assuming a monomer–tetramer equilibrium. Uncertainties due to spectral subtraction, compounded with low concentrations and the exponential character of the dissociation constant, lead to the large range. Spectral subtraction separates two components of a composite spectrum by subtracting a single component from the composite spectrum. In this case, the spectrum of 220 μM hr.m was assumed to be completely oligomeric (theoretically it is 98% oligomerized) and subtracted from composite spectrum. The difference EPR spectrum was sharp, as expected for a random-coil peptide (Schneider & Freed, 1989; Thorgeirsson *et al.*, 1995). The desired difference spectrum was not exactly known. This led to a range of possible compositions, which are reflected in the reported errors. If too much was subtracted, the difference spectrum would erroneously have a negative first derivative (corresponding to microwave emission) before each of the three absorbance lines. The spin concentration of each species was calculated from double integration.

In order to test the hypothesis that the coiled-coil region is involved in membrane binding, these peptides were mixed with lipid vesicles and studied by EPR. Conservative hr mutants were bound to POPC vesicles containing 10 mol % POPG (Figure 6). This is evidenced by a large broadening of the EPR spectrum indicative of τ_R in the range 3–4 ns. The spectra are similar to those for spin-labeled melittin bound to vesicles (Altenbach & Hubbell, 1988).

The hyperfine splitting of nitroxide spin labels is sensitive to the polarity of its environment (Seelig & Hasselbach, 1971). In nonpolar environments, such as the acyl chain region of lipid bilayers, the hyperfine splitting decreases by as much as 3 MHz, corresponding to 1 G on an X-band EPR spectrometer. When the conservative hr mutants bound to lipid vesicles, the hyperfine splitting remained the same,

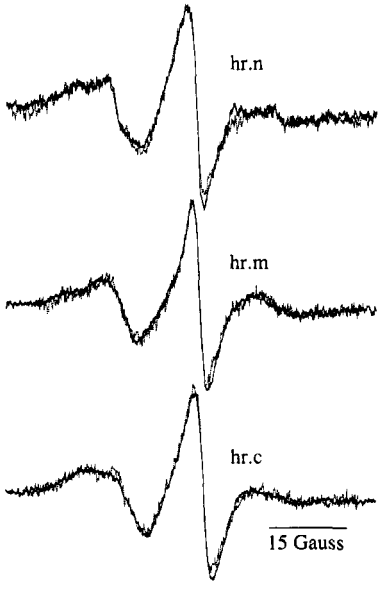

FIGURE 7: EPR spectra of hr.n, hr.m, and hr.c bound to 10 mol % POPG vesicles, with (solid line) and without (dashed line) hr.wt present. All samples are in 20 mM MOPS at pH 7.2. All samples without hr.wt have 50 μM peptide. With hr.wt present, hr.n was 23 μM, hr.wt was 80 μM, and the total lipid was 48 mM; with hr.wt present, hr.m was 21 μM, hr.wt was 60 μM, and the total lipid was 32 mM; and with hr.wt present, hr.c was 19 μM, hr.wt was 54 μM, and the total lipid was 32 mM.

indicating that the polarity of the spin-label environment did not change. This indicates the spin label is not buried in the acyl chain region of the bilayer. The peptide hr.n is labeled near the amino terminus, hr.m near the middle, and hr.c near the carboxyl terminus. They all have similar EPR spectra, indicating that they are all in similar environments, most likely near the water−bilayer interface, and suggesting that the peptide binds parallel to the membrane surface.

As seen in the solution spectra (Figure 3), oligomerization can be detected by adding unlabeled peptide because proximate spin labels interact with each other. When a mixture of unlabeled hr.wt and labeled hr.n, hr.m, or hr.c is added to lipid bilayers, the spectral line widths are the same as when hr.wt is not present (Figure 7). This indicates that the spin labels of different peptide subunits in an oligomer are separated when bound to vesicles, suggesting that the peptide tetramer dissociates into monomers upon insertion into lipid bilayers.

The second class of mutations, disruptive mutations, consisted of replacement of isoleucine 573 with aspartate (hr.D), serine (hr.S), or proline (hr.P). These mutations are expected to disrupt coiled-coil formation because they are at the "a" position, which is part of the coiled-coil hydrophobic contact area (Crick, 1953; Chothia *et al.*, 1977; O'Shea *et al.*, 1991). Dubay *et al.* (1992) have shown that mutations at these positions do not affect gp41 oligomerization in the prefusogenic state but they do inhibit virus infectability. On the basis of the mean residue elipticities at 222 nm ($[\theta_{222}]$) from CD spectroscopy (Figure 2), hr.D and hr.P are 16% ± 4% helical at 10 μM and hr.S is 21% ± 4% helical at 10 μM (Chen *et al.*, 1974; Greenfield & Fasman, 1969), indicating that these mutations do indeed disrupt coiled-coil formation. These peptides also contain a cysteine in place of the tryptophan at the f position (W571C) in the middle of the peptide for nitroxide spin labeling. This mutation did not affect helicity in hr.m (Figure 2), so we expect that the same mutation is not structurally significant in hr.D, hr.P, and hr.S. Furthermore, CD spectra of spin-labeled hr.P and hr.S agree with those of hr.P and hr.S without the W571C mutation (Wild *et al.*, 1994).

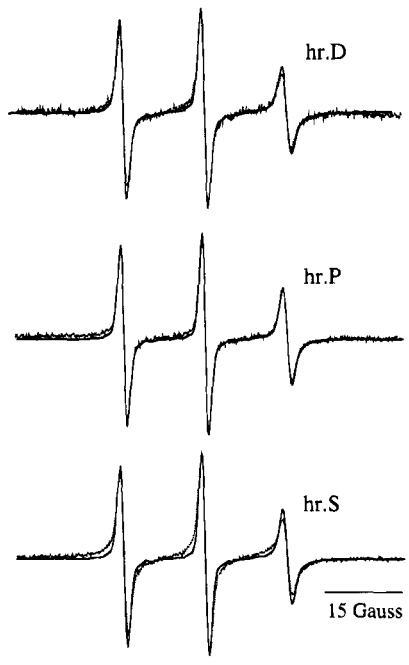

FIGURE 8: EPR spectra of 100 μM hr.D, hr.P, and hr.S in 20 mM MOPS, pH 7.2 (solid line), and of 50 μM hr.D, hr.P, and hr.S with 300 μM hr.wt (dashed line).

The solution EPR spectra of peptides hr.S, hr.P, and hr.D are very narrow with τ_R in the range 0.3−0.7 ns (Figure 8). These spectra are consistent with peptides predominantly in the random-coil, not coiled-coil, state. Using EPR spectral subtraction as before, hr.P and hr.S were found to be 17% ± 3% oligomerized at 50 μM and hr.S was found to be 24% ± 3% oligomerized at 50 μM. Although the concentrations are not exactly the same, this is consistent with CD measurements (Figure 2). When 6-fold excess unlabeled hr.wt is added, the oligomeric component of the spectrum of hr.S increases. This indicates that a fraction of the hr.S peptides form heteromeric coiled coils with wild-type peptide. The peptides hr.P and hr.D show this behavior to a lesser extent (Figure 8).

As seen in Figure 9, the peptides hr.S, hr.P, and hr.D do not appreciably bind to vesicles containing 10% POPG. These peptides have a net positive charge. In order to increase the binding to negatively charged vesicles, the membrane surface charge was increased by using vesicles with more POPG. The mutants hr.S and hr.P required 20% charge for appreciable binding (99% and 95%, respectively). Furthermore, at 30% charge the mutant hr.D was only 65% bound. The membrane-binding affinities correlate with the coiled-coil propensities. These results are summarized in Table 2.

DISCUSSION

The envelope TM glycoproteins of both HIV-1 and influenza viruses contain leucine zipper-like heptad repeats close to their fusion peptides (Gallaher *et al.*, 1989). This region is crucial to the virus life cycle, and understanding its specific role may help understand the mechanism of membrane fusion.

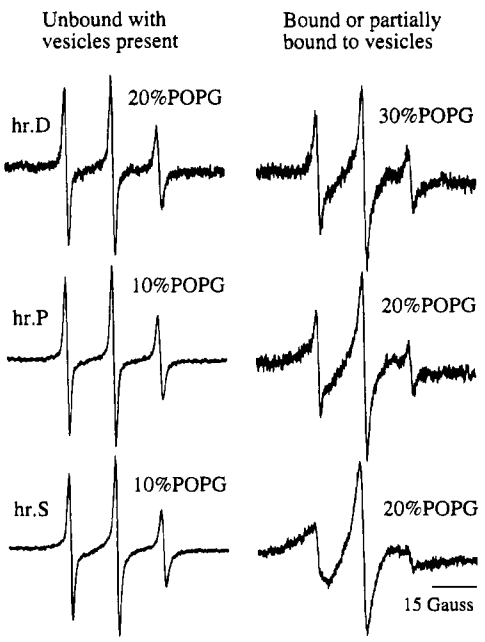

FIGURE 9: EPR spectra of 50 μM hr.D, hr.P, and hr.S in 20 mM MOPS, pH 7.2, in the presence of lipid vesicles composed of POPC and POPG. The mole percent of POPG is indicated. The total lipid concentration was 48 mM.

Table 2: Correlation between Coiled-Coil Propensity and Membrane-Binding Affinity

hr.n	+++	+++
hr.m	+++	+++
hr.c	+++	+++
hr.S	+	++
hr.P	+	++
hr.D	+	+

[a] "+ + +", spontaneous coiled-coil formation; "+", decreased coiled-coil formation. [b] "+ + +", binding to vesicles with 10% negative charge; "+ +", 20% negative charge; and "+", 30% negative charge.

Peptides derived from the heptad repeat region of both HIV gp41 and influenza HA2 have been found to form coiled coils (Wild *et al.*, 1992; Carr & Kim, 1993). Heptad repeat regions generally participate in oligomerization by forming intermolecular coiled coils, such as the GCN4 leucine zipper (O'Shea *et al.*, 1991) and influenza virus HA2 (Wilson *et al.*, 1981). The heptad repeat region of native gp41, however, does not appear to be responsible for oligomerization before CD4 binding (prefusogenic state). It was found that with coiled-coil disrupting mutations, the protein was still transported to the viral membrane correctly. Furthermore, its oligomerization, as determined by equilibrium sedimentation ultracentrifugation, was not affected (Dubay *et al.*, 1992; Chen *et al.*, 1993). This suggests that coiled-coil formation is not essential for correct folding in the prefusogenic state.

The heptad repeat region, however, is crucial to the protein's function. Mutations in this region stop virus infectability, as detected by a lack of virus-mediated cell–cell fusion of HIV infected cells (Dubay *et al.*, 1992; Chen *et al.*, 1993). Other evidence suggests that coiled-coil formation is somehow important to the protein's function. First, hr.wt (also known as dp107), a 38-residue peptide from the heptad repeat region, oligomerizes and forms a tetrameric coiled coil. In fact, at 37 °C it is 85% helical (Wild *et al.*, 1992). Thus, if structural constraints preventing coiled-coil formation are removed, it will spontaneously form a tetrameric coiled coil. Receptor binding may induce such a conformational change. Secondly, 5 μg/mL of this peptide added to virus particles inhibit infectability. This may be because in the fusogenic state the peptide competes with the native protein for oligomerization, thereby disrupting protein oligomerization. Finally, when two of these peptides were linked by a disulfide bond, they became a more potent inhibitor, requiring only 1 μg/mL. This further suggests that inhibition occurs by disrupting oligomerization, as a disulfide-linked dimeric peptide would have a higher affinity than a monomer for oligomerization if the oligomerization state is trimeric or higher.

It has been suggested that the heptad repeat region of gp41 is not a coiled coil in the prefusogenic state (Dubay *et al.*, 1992, Chen *et al.*, 1993; Wild *et al.*, 1994). Instead, upon binding to CD4, the protein changes conformation to the fusogenic state and the heptad repeat region becomes an extended coiled coil which presents the fusion peptide to the host-cell membrane. In support of this model, a conformational change in the gp160 complex upon CD4 binding has been observed (Sattentau & Moore, 1991). This model is similar to the proposed "spring-loaded" mechanism of influenza hemagglutinin (Carr & Kim, 1993). In contrast to HIV gp41, influenza virus hemagglutinin induces membrane fusion at low pH. A heptad repeat region of hemagglutinin changes from loop to coiled coil upon acidification. This positions the fusion peptide as much as 100 Å away from the viral membrane so it can interact with the host-cell membrane (Carr & Kim, 1993; Bullough *et al.*, 1994). Analogously, immediately after CD4 binding, the function of the heptad repeat region of gp41 may be to form an extended coiled coil which presents the fusion peptide to the target membrane. Our results suggest that the heptad repeat region of gp41 forms a tetrameric coiled coil in the fusogenic state.

When the fusion peptide binds to the host-cell membrane, however, the two membranes are still separated by the long coiled coil. If no other part of the TM protein were to bind to the host-cell membrane, fusion would not be completed. However, fusion would be facilitated by the insertion of the extracellular domain of the TM into the host-cell membrane.

Evidence suggests that the heptad repeat region of HA2 actively participates in membrane fusion by binding to the host-cell membrane in addition to bringing the fusion peptide close to the host cell. Yu *et al.* (1994) found that a peptide from the heptad repeat region bound to negatively charged vesicles following acidification. In this study, we show that the heptad repeat region of gp41 also binds to negatively charged lipid vesicles (Figure 8). Thus, this region of the protein gp41 may facilitate membrane fusion by binding to the host-cell membrane.

Amphiphilic helices have both hydrophobic and polar sides. Coiled coils have a hydrophobic core formed by nonpolar residues at the "a" and "d" positions, so a monomeric α-helical peptide from a coiled coil is amphiphilic. This is suitable for interacting with membranes because the hydrophobic face can interact with the aliphatic portion of the lipids while the polar groups prefer to be in a

polar environment. As an example, amphiphilicity has been generally found in the N-terminal regions of membrane-binding mitochondrial signal peptides (von Heijne, 1986) and melittin (Terwilliger & Eisenberg, 1982).

The amphiphilicity of hr.wt is seen in Figure 1. Its hydrophobic surface was disrupted by the introduction of the less hydrophobic residues at the "a" position. Furthermore, proline is expected to break the helix and thus disrupt the hydrophobic surface. These mutations decreased both membrane-binding affinity and coiled-coil formation (Table 2). Thus, the heptad repeat region requires a hydrophobic surface for both membrane binding and coiled-coil formation. Analogous mutations in gp41 stopped syncitia formation *in vivo* (Dubay *et al.*, 1992). Thus, the *in vivo* results may be due to decreased gp41 target membrane binding as well as lack of coiled-coil formation in the fusogenic state.

According to a simple model based on Gouy−Chapman electrostatic theory, the binding of a peptide can be separated into electrostatic and nonelectrostatic components:

$$K_{bind} = K_o\, e^{-ZF\Psi/RT} \quad (1)$$

where K_{bind} is the total binding constant, K_o is the nonelectrostatic binding constant, Z is the net peptide charge, F is Faraday's constant, Ψ is the membrane surface potential, **R** is the universal gas constant, and T is the temperature (McLaughlin, 1989; Thorgeirsson *et al.*, 1995). The exponential term that represents the electrostatic component is due to the attraction (or repulsion) between the peptide charge and the membrane surface charge. K_o, the nonelectrostatic component, is due to all other effects such as hydrophobic effects, solvation, and hydrogen bonding. Thus, peptides with the same charge, but with different nonelectrostatic binding affinities (K_o), can be made to bind equally by adjusting the membrane surface charge (Ψ).

All hr mutants have a net charge of +2, except for hr.D, which has a net charge of +1. The observation that a more negatively charged vesicle is necessary for membrane binding indicates that hr.S and hr.P have a smaller K_o and thus lower nonelectrostatic affinities for membranes than hr.n, hr.m, and hr.c. The decreased binding is most likely due to disruption of the hydrophobic face.

Binding of the conservative hr peptides to lipid vesicles suggests a role for the heptad repeat region of gp41. If this region of the whole protein were to bind to the host cell during fusion, it would bring the two membranes closer together, presumably accelerating fusion. Furthermore, it is also shown that the peptide is most likely monomeric in the membrane-inserted state. This suggests that the tetrameric coiled coil uncoils upon membrane binding so that the hydrophobic face can be solvated by the phospholipid. Thus, upon CD4 binding, the heptad repeat region of the protein may briefly form a coiled coil to present the fusion peptide to the target membrane. The coiled coil then uncoils, bringing the two membranes closer. This is analogous to the mechanism proposed by Yu *et al.* (1994) for hemagglutinin-induced membrane fusion.

In summary, it is shown that amphiphilicity is required for both membrane binding and coiled-coil formation of the heptad repeat peptides. Combining this information with previous studies leads to a model in which the heptad repeat region is not a coiled coil in the prefusogenic state. After CD4 binding, the heptad repeat region forms a coiled coil and brings the fusion peptide close to the cellular membrane. Next the fusion peptide inserts into the cellular membrane, which is followed by binding of the heptad repeat. This model provides two reasons as to why lethal mutations in the heptad repeat region stop fusogenic activity: (1) they disrupt coiled-coil formation in the fusogenic state and (2) they decrease the membrane-binding ability of the protein.

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