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The Antiviral Activity of a Synthetic Peptide Derived from the Envelope SU Glycoprotein of Feline Immunodeficiency Virus Maps in Correspondence of an Amphipathic Helical Segment

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In a previous paper (Lombardi *et al., Virology* 220, 274–284, 1996), we reported that a 20-amino acid synthetic peptide derived from a conserved region of the SU glycoprotein of feline immunodeficiency virus (FIV), i.e., ^{225}EGPTLGNWAREIWATLFKKA^{244}, bound the surface of FIV-permissive cells and inhibited FIV infection of CrFK and lymphoid cells. In this paper, we report, by the use of N- and C-terminus deleted synthetic analogs and by glycine scanning experiments that the minimal sequence needed for the full antiviral activity of the peptide maps in correspondence of amino acids ^{229}LGNWAREIWATL^{240} and that either tryptophans residues at sequence position 232 or 237 are essential for such activity. Circular dichroism (CD) studies indicate that in the presence of a hydrophobic environment the ^{225}E-A^{244} peptide adopts a structure containing an amphipathic α-helical segment of approximately 7 residues, corresponding to 2 helical turns, likely in correspondence of the sequence ^{231}(N)WAREIW(A)^{238}. Such a helical segment of FIV SU glycoprotein may play a role in viral envelope fusion role with the host cell membrane, thus proving critical for cell infection.

Feline immunodeficiency virus (FIV) is a widespread lentivirus of domestic cats sharing numerous biological and pathogenetic features with the human immunodeficiency virus (HIV). FIV infection in cats has been therefore proposed as an animal model for AIDS studies with respect to pathogenesis, chemotherapy and vaccine development (1-4).

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Similar to HIV, FIV infects T lymphocytes and FIV-infected cats show a marked reduction of circulating $CD4^+$ T lymphocytes (5,6); cells of the monocyte-macrophage-microglia lineage and astrocytes are also infected *in vitro* (1,7,8). Despite these similarities - which led to the initial suggestion that, as for HIV, the feline CD4 (fCD4) antigen might be the cellular receptor for FIV -, there is a lack of correlation between the expression of fCD4 on cells and their ability to support FIV replication (6). The feline cell surface marker CD9, identified by means of the cell-specific monoclonal antibody vpg15 that efficiently blocks FIV infection of susceptible cells *in vitro*, was proposed as a putative non-CD4 receptor or co-receptor (9,10); blocking of FIV infection by vpg15, however, was subsequently related to inhibition of virus release rather than interference with receptor binding (11). Recently, it has been shown that FIV, similar to HIV and simian immunodeficiency virus (12,13), uses the α-chemokine receptor CXCR4 for cell fusion, thus suggesting a common mechanism of infection by lentiviruses (14,15).

On the other hand, little information is available on the Env domains involved in FIV binding to target cells and on the other physiological events underlying FIV attachment and penetration. The third variable region (V3) of the Env surface (SU) glycoprotein seems to be a primary determinant for FIV binding to and entering susceptible cells (16,17), but other Env domains have been postulated. In a previous paper we reported that peptides derived from two regions of the *env* gene product of the 34TF10 clone of the Petaluma isolate of FIV (FIV-Pet) (18) exerted a remarkable and specific antiviral activity *in vitro*. More particularly, peptides from one of these domains, spanning residues ^{225}E-P^{264} of a conserved region of the SU glycoprotein bound the sur-

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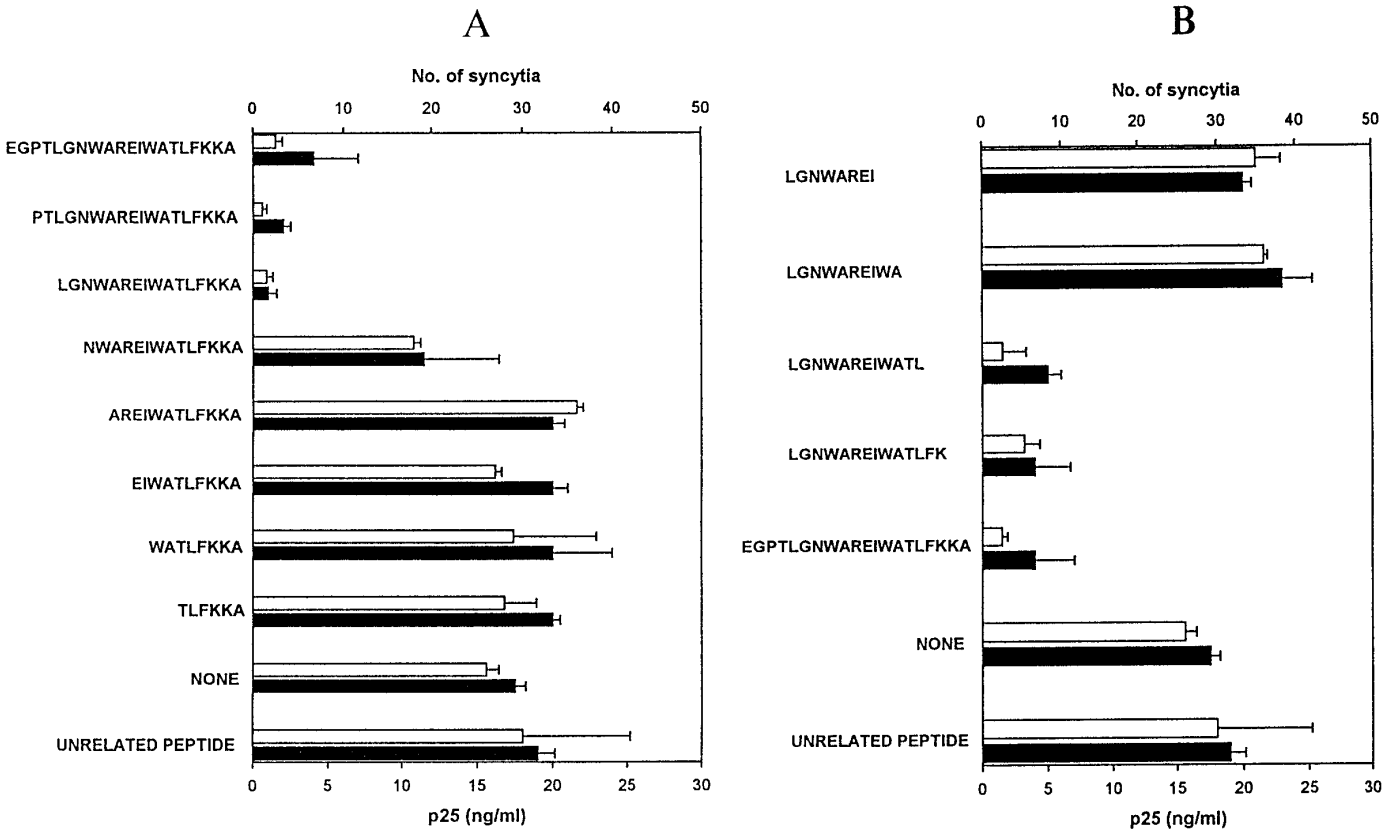

FIG. 1. Effect of (A) N-terminus and (B) C-terminus deleted analogs of peptide 5 on FIV infection of CrFK cells. An unrelated 20-amino acid synthetic peptide (previously coded 66 [19]) from FIV-Pet Env was used as a non-inhibitory negative control. □, numbers of syncytia; ■, p25 antigen levels. Each bar represents the mean + S.D. of triplicate assays.

face of FIV-permissive cells and inhibited FIV infection of CrFK and lymphoid cells. Circular dichroism (CD) analysis showed that a 20-amino acid synthetic peptide derived from this domain, ^{225}EGPTLGNWAREIWATLFKKA^{244}, named peptide 5, folds to a helical conformation in the presence of a hydrophobic environment. We suggested that the SU peptide might specifically interact with cell surface molecules involved in viral infection and compete with FIV binding to cell receptor(s) (19).

In this paper, by the use of N- and C-terminus deleted synthetic analogs and by glycine scanning experiments we have mapped the determinants of antiviral activity of peptide 5; moreover, CD analysis of selected synthetic analogs has shown that the antiviral activity of peptide 5 maps in correspondence of an amphipathic helical segment.

MATERIALS AND METHODS

Virus, cells and culture conditions. The California isolate Petaluma (FIV-Pet), produced by chronically infected FL4 cells kindly provided by Dr. J. Yamamoto (20,21) has been used. CrFK cells were grown in Eagle's minimal essential medium supplemented with 0.5% fetal bovine serum (FBS) and additional supplement (22).

Synthetic peptides. Peptite 5 and its glycine-scanned analogs were manually synthesized using N-α-fluorenylmethoxycarbonyl (FMOC)-protected amino acids and a p-alkoxy-benzyl alcohol resin as solid phase, as previously described (23). The N- and C-terminus deleted analogs of peptide 5 were prepared by continuous-flow solid-phase peptide synthesis using conventional FMOC-strategy on a Milligen 9050 automatic synthesizer and purified to homogeneity by preparative reverse-phase high-pressure liquid chromatography (HPLC). The identity of the final products, whose HPLC purity was greater than 97%, was verified by electrospray mass spectrometry.

Peptide inhibition of syncytium formation. Peptides were screened for the capability to inhibit FIV replication using an assay based on FIV-induced syncytium formation (22). Briefly, solutions of peptides were added at the final concentration of 16 μg/ml to 2 x 10^4 CrFK cells in 0.8 ml of culture medium containing 0.5% FBS in 24-well plates; after 1 hr at 37°C approximately 50-100 syncytium forming units (SFU) of FIV-Pet were added in 0.2 ml-volume. Six days later, the cultures were stained and the number of was syncytia counted under the microscope.

Capture ELISA for p25 antigen. FIV p25 core antigen was assayed by capture ELISA as previously described (24). Briefly, plates (Probind, Falcon, Italy) were coated overnight with 0.5 μg purified AE11 MAb in 100 μl carbonate buffer pH 9.6. After 4 washes with PBS containing 0.05% Tween 20 (PBS-Tw), plates were post-coated with 150 μl of PBS containing 1% bovine serum albumin (BSA) (PBS-BSA) for 1 hr. Test samples (100 μl) containing 0.5% Triton X-100 were added to the wells and incubated for 2 hr. After 4 washes, 0.1 μg biotin-conjugated DF10 MAb in 100 μl of PBS containing 1% skim milk, 5% fetal calf serum and 0.05% Tween 20 (dilution buffer) were

added and incubated 1 h. The plates were then washed and further incubated 1 hr with 100 μl of a horseradish peroxidase (HPRO)-conjugated anti-biotin (Sigma, St. Louis, Mo) diluted 1:1,000 in PBS-Tw-BSA. The enzyme reaction was carried out with 100 μl tetramethylbenzidine (KPL, Gaithersburg, MD) and stopped with 50 μl 0.1 N H_2SO_4; the absorbance was measured at 450 nm. All steps were performed at r.t.. Duplicate wells containing two-fold dilutions of recombinant p25 (kindly provided by Dr. O. Jarrett) ranging from 500 ng/ml to 0.03 ng/ml served for standard curve.

Circular dichroism (CD) measurements. CD spectra were recorded at 25°C on a Jasco J500A spectropolarimeter in 10 mM sodium phosphate buffer, pH 7.05, containing 150 mM NaCl or in a solvent mixture containing 10% of the same buffer and 90% methanol (v/v). In the peptide region (200-250 nm), data are expressed as molar ellipticity [Θ] (deg cm^2 $dmol^{-1}$) based on the mean residue molecular weight; in the aromatic region (250-320 nm), ellipticity [Θ] is based on the peptide molecular weight and is given per mole of tryptophan.

RESULTS

Antiviral activity of deleted analogs of peptide 5. As a first step to map the determinants of antiviral activity of peptide 5, a number of analogs, each characterized by progressive deletions of 2 amino acids at the N terminus, were synthesized and purified to homogeneity. These peptides were compared with the parent compound for their ability to inhibit FIV infection of CrFK cells. Each peptide was added to CrFK cells in 24-well plates and after 1 hr at 37°C the cultures were inoculated with 50-100 SFU of FIV-Pet. FIV-induced syncytia and FIV p25 core antigen in culture supernatants were determined 6 days later. As a non-inhibitory negative control a 20-amino acid synthetic peptide (previously coded # 66 [19]) from FIV-Pet Env was used. Figure 1A summarizes the results of one representative experiment out of three. As expected, parent peptide 5 exhibited a marked antiviral activity, both in terms of reduction of syncytium formation and p25 release; peptides with 2 and 4 residue deletions maintained an inhibitory activity similar to that of the parent compound. The peptide with N terminus deletion of 6 amino acids induced a slight reduction in syncytium formation and p25 antigen release, while analogs with 8 to 14 residue deletions exhibited a dramatic loss of antiviral activity. To map the minimal active sequence of the antiviral peptide 5, a further series of analogs, each characterized by the deletion the first 4 N-terminus amino acids, i.e. EGPT, and progressive deletions of 2 amino acids at the C terminus, were synthesized and tested for antiviral activity as described above. Figure 1B summarizes results of experiments in which each peptide was tested at least twice. As shown, analogs with 2 and 4 amino acids deletions at the C-terminus maintained an inhibitory activity similar to that of the parent compound, while peptides with C-terminus deletions of 6 or more residues exhibited a complete loss of antiviral activity. Residues ^{229}LGNWAREIW-ATL^{240} are therefore needed for the full antiviral activity of the peptide.

TABLE 1

Effect of Glycine Scanning on the Antiviral Activity of Peptide 5

Peptide No.	Residue substituted with glycine	No. of syncytia[a]	p25 (ng/ml)[a]
None		48.0 ± 5.3	33.0 + 2.5
Unrelated peptide[b]		39.0 ± 3.6	27.0 + 3.2
5		0.0 ± 0.0	<0.25
225	E^{225}	0.0 ± 0.0	<0.25
227	P^{227}	0.0 ± 0.0	<0.25
228	T^{228}	0.0 ± 0.0	<0.25
229	L^{229}	0.0 ± 0.0	<0.25
231	N^{231}	0.0 ± 0.0	<0.25
232	W^{232}	30.0 ± 1.5	25.0 + 2.1
233	A^{233}	0.0 ± 0.0	<0.25
234	R^{234}	3.0 ± 2.0	7.0 + 0.5
235	E^{235}	0.3 ± 0.6	<0.25
236	I^{236}	0.0 ± 0.0	<0.25
237	W^{237}	32.0 ± 7.0	30.0 + 3.8
238	A^{238}	0.3 ± 0.6	<0.25
239	T^{239}	0.0 ± 0.0	<0.25
240	L^{240}	0.0 ± 0.0	<0.25
241	F^{241}	0.0 ± 0.0	<0.25
242	K^{242}	0.0 ± 0.0	<0.25
243	K^{243}	0.0 ± 0.0	<0.25
244	A^{244}	0.0 ± 0.0	<0.25

[a] Data expressed as means ± S.D. See legend to Fig. 1 for details on the assay.

[b] As a non-inhibitory negative control, an unrelated 20 amino acid synthetic peptide (previously coded 66 [19]) from FIV-Pet Env was used.

Glycine scanning of peptide 5. In a different approach to map the determinants of antiviral activity of peptide 5, we prepared 18 peptides of 20 amino acids, in which each residue (other than glycine) of the sequence of parent peptide 5 was substituted with the conformationally flexible glycine residue (glycine scanning). Such compounds were compared with the parent peptide 5 for their ability to inhibit FIV infection in CrFK cells by determining FIV-induced syncytia and p25 at 6 days after infection. Results of a representative experiment are shown in Table 1. As shown, glycine substitution for either tryptophans at sequence position 232 or 237 determined an almost complete loss of antiviral activity, whereas glycine substitutions for all the other residues were absolutely ineffective.

CD analysis of selected peptides. Parent peptide 5, peptides 232 and 237 (in which glycine substitution for W^{232} and W^{237}, respectively, determined the loss of biologic activity), and peptides 227, 233, 236 and 241 (in which a glycine residue was substituted at positions P^{227}, A^{233}, I^{236}, and F^{241}, respectively, with no loss of biologic activity) were analyzed by CD spectroscopy; measurements were carried out in sodium phosphate buffer or in the same buffer in the presence of 90% methanol. In the absence of methanol (Figs. 2a and 2b),

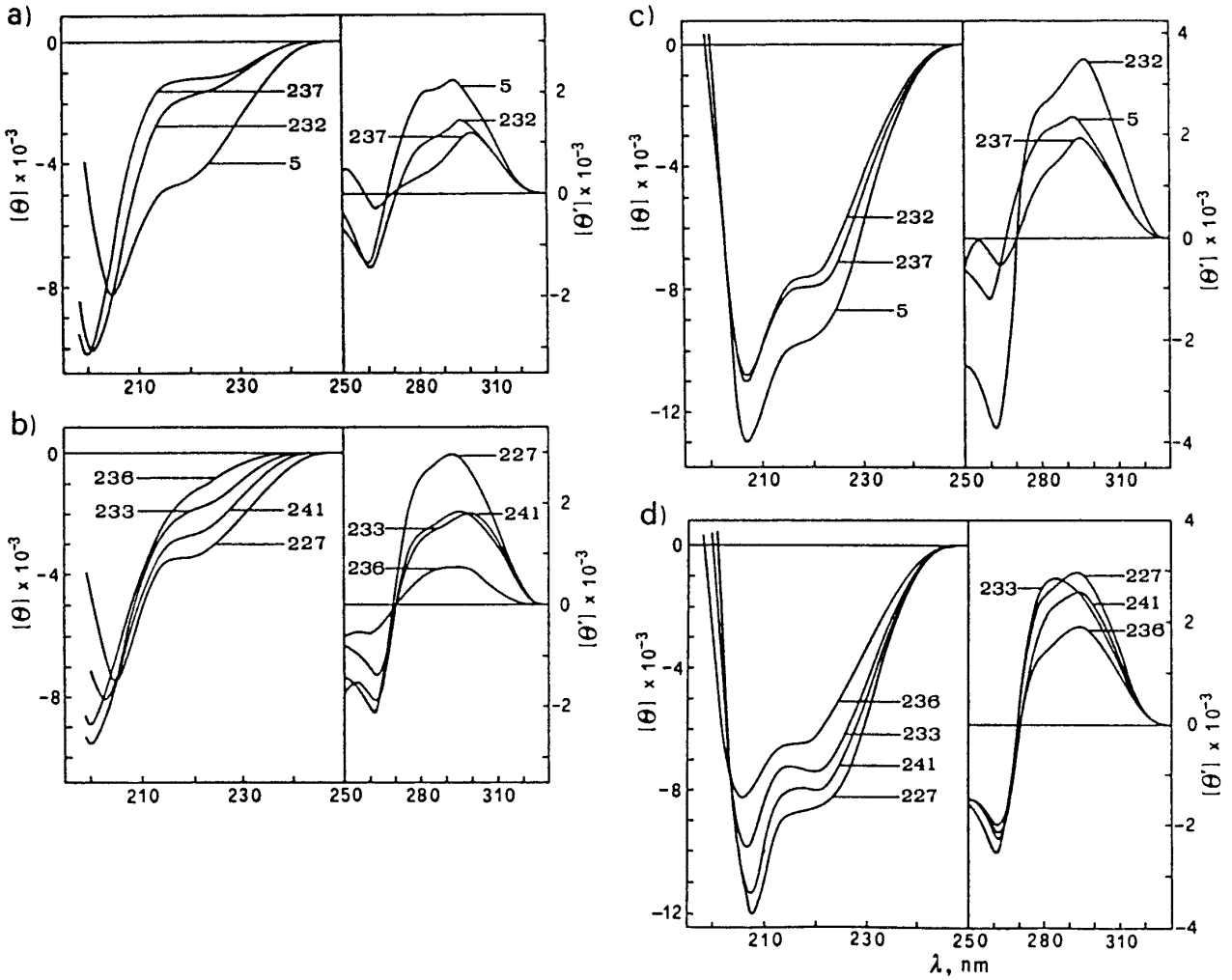

FIG. 2. Circular dichroism (CD) spectra of peptides 5, 232, and 237 (panels a and c) and peptides 227, 233, 236, and 241 (panels b and d). CD measurements were made in 10 mM sodium phosphate buffer, pH 7.05, containing 150 mM NaCl (panels a and b) or in a solvent mixture containing 10% of the same buffer and 90% methanol (v/v) (panels c and d). The spectra were found to be independent of peptide concentration in the range of $8x10^{-2}$ to $8x10^{-1}$ g/l, thus showing that in this range the peptide molecules are monomeric and do not aggregate in solution.

the CD spectra of peptides 5, 227, and 241 showed a weak band at about 220 nm, thus suggesting the presence of a very small amount (10-15%) of helical structure. On the other hand, the spectra of peptides 232, 237, 233, and 236 indicated that all these peptides adopt a random coil structure, as usually expected for short peptides. In the presence of 90% methanol (Figs. 2c and 2d), the spectra of all peptides exhibited the two negative minima at 222 and 208 nm, typical of the α-helix structure. In the case of parent peptide 5, assuming $[\Theta]_{222}$ = ca. -30,000 deg cm^2 dmol^{-1} for a 100% helical structure containing 19 peptide chromophores at 25°C (25-27), the intensity of the band at 222 nm, $[\Theta]_{222}$ = -11,000 deg cm^2 dmol^{-1}, corresponds to a helix content of about 35%; this suggests that approximately 7 residues of the peptide sequence form two α-helix turns, as already reported (19). The other peptides showed CD spectra quite similar to peptide 5, although of slightly weaker intensity. The weak CD bands in the near-UV region were quite similar for all the peptides, both in the absence and in the presence of methanol, so they can be attributed to the intrinsic contribution of tryptophan residues, independently of the peptide conformation. Possible contributions, if any, of aromatic residues in the peptide region should be very small, as indicated by the typical CD pattern of partially helical short peptides.

By comparing the CD spectra of the studied peptides in methanol/buffer solution (Figs. 2c and 2d), it can be observed that *(a)* substitutions G → P^{227} (peptide 227) and G → F^{241} (peptide 241) do not induce significant variations in CD spectra compared to peptide 5, thus indicating that both P^{227} and F^{241} are not inserted in the helical domain; *(b)* CD spectra of peptides 232, 233,

236 and 237 in which the helix-destabilizing glycine residue was substituted to W^{232}, A^{233}, I^{236} and W^{237}, respectively, showed significant reduction of helical structure, thus suggesting that these four residues are likely included in the helical domain. Taken all together, the results of CD analysis suggest that in polar conditions peptide 5 adopts an essentially random coil structure, whereas in a less polar environment, such as in methanol solution, it adopts a structure containing an α-helical segment of approximately 7 residues, corresponding to two helical turns, likely in correspondence of the sequence ^{231}(N)-W-A-R-E-I-W-(A)^{238}.

DISCUSSION

In a previous paper (19), we reported that a 20-amino acid synthetic peptide, coded peptide 5, derived from a conserved region of the SU glycoprotein of FIV, *i.e.* ^{225}EGPTLGNWAREIWATLFKKA^{244}, bound the surface of FIV-permissive cells and inhibited FIV infection of CrFK and lymphoid cells; in the presence of a hydrophobic environment approximately 7 residues of the peptide sequence form two α-helix turns.

In this paper, by the use of N- and C-terminus deleted synthetic analogs and by glycine scanning experiments, we show that the minimal sequence needed for the full antiviral activity of peptide 5 maps in correspondence of amino acids ^{229}LGNWAREIWATL^{240} and that either tryptophans residues at sequence position 232 or 237 are essential for such activity. Moreover, the results of CD analysis suggest that in an apolar environment, as it may occur in the presence of a cell membrane, it adopts a structure containing an α-helical segment of approximately 7 residues, corresponding to two helical turns, likely in correspondence of the sequence ^{231}(N)-W-A-R-E-I-W-(A)^{238}. The helical segment detected by CD analysis, schematically represented in Fig. 3, possesses the characters of an amphipathic α-helix and it is noteworthy that, although glycine substitution for any amino acid residue did not determine significant conformational changes, at least in the analogs studied by CD spectroscopy, substitution of either tryptophans, i.e. the amino acids that proved important for the biological activity of peptide 5, produces a marked variation in the amphipathy of the helix.

Thus, the results obtained with deleted and glycine-scanned analogs of peptide 5 and those obtained by CD analysis point to the conclusion that the antiviral activity of peptide 5 maps in correspondence of a central sequence with the characters of an amphipathic α-helix, in which the tryptophan residues play a critical role. In fact, the highly lipophylic tryptophans are positioned approximately at the opposite sides of the hydrophobic face, thus contributing both to the amphipathy of the helix and, likely, to insertion into the cell membrane. Amphipathic α-helix conformation is known

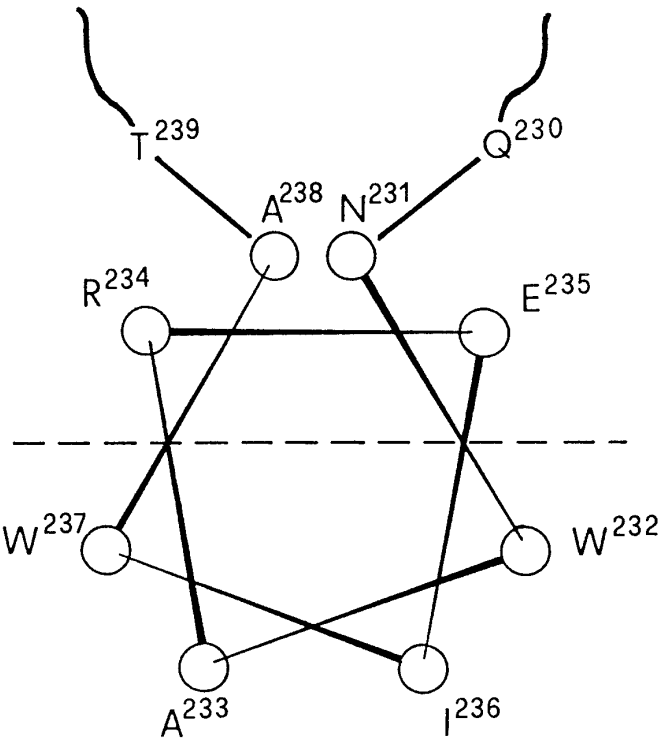

FIG. 3. Schematic wheel representation of the amphipathic α-helical segment of peptide 5.

to be shared by peptides endowed with cytolytic activity produced by different organisms (28,29), as well as by cytolytic synthetic peptides derived from the cytoplasmic domain of HIV-1 gp41 (30,31). In general, the interaction between the amphipathic segment of the cytolytic peptide and cell membrane generates ion channels in the lipid bilayer thus leading to increase in cell permeability and cell lysis (32-35). Although peptide 5 and its deleted analogs are not cytolytic over a wide range of concentration (unpublished observations), it can be hypothesized that the corresponding amphipathic helix domain on the SU glycoprotein of FIV may play a role in fusion of the viral envelope with cell membranes. In fact, in an assay in which mixing of FIV-infected lymphoid FL4 cells with CrFk cells results in cell-cell fusion within 24 hours, peptide 5 completely inhibited syncytium formation and, by using the deleted analogs described above, also this effect was found to map in correspondence of the segment ^{229}LGNWAREIWATL^{240} (data not shown). These data support, therefore, the idea that the inhibitory peptide may act at an early stage of cell infection, likely by interacting with cell surface molecules involved in virus attachment and/or penetration.

Finally, the amphipathic α-helix conformation of peptide 5 deserves further attention, as it is known that amphipathic α-helical protein sequences tend to be T-cell epitopes (36). Peptide 5 actually lacks antibody-

inducing-activity; we have in fact observed that peptide 5 is not recognized by sera of cats infected with FIV-Pet or with a local FIV isolate (FIV-M2) and does not induce detectable humoral responses when repeatedly injected into mice (37). Peptide 5 or an active analog might therefore prove important in future studies as a component of a candidate vaccine for directing host's T-cell responses to a biologically important site of FIV SU glycoprotein.

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