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Inhibition of Feline Immunodeficiency Virus Infection *in Vitro*

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Sixty-six 20- to 23-amino-acid synthetic peptides, partially overlapping by 10–12 amino acids, spanning the entire sequence of the envelope SU and TM glycoproteins of the Petaluma isolate of FIV, have been used to investigate the Env domains involved in viral infection. Peptides 5 to 7, spanning amino acids ^{225}E–P^{264} located in a conserved region of the SU protein, and peptides 58 to 61, spanning amino acids ^{757}N–P^{806} and encompassing hypervariable region 8 of TM protein, exhibited a remarkable and specific antiviral effect against the homologous and one heterologous isolate, as judged by inhibition of FIV-induced syncytium formation and p25 production in CrFK cells. Peptides 5 and 7, but not peptides 58 and 59, also inhibited viral replication of a fresh FIV isolate on nontransformed lymphoid cells. By flow cytometry, peptides 5, 7, 58, and 59 were shown to bind the surface of FIV permissive cells. The antiviral activity of peptides 5 and 7, however, was time-dependent, as inhibition of FIV replication was seen when the peptides were administered before or within 3 hr after virus inoculation; in contrast, TM peptides 58 and 59 exerted a potent inhibitory effect when added up to 24 hr after virus inoculation. Circular dychroism analysis showed that peptide 5 folds to a helical conformation in the presence of a hydrophobic environment. Although the basis for the antiviral action of the peptides is not understood, our data suggest that the inhibitory peptides may act by interacting with cell-surface molecules involved in viral infection.

INTRODUCTION

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 therefore been proposed as an animal model for AIDS studies with respect to pathogenesis, chemotherapy, and vaccine development (Pedersen, 1993; Bendinelli *et al.,* 1995).

Similar to HIV and other lentiviruses, the FIV envelope (Env) surface protein (SU) gp95 and the transmembrane protein (TM) gp40, encoded by the *env* gene, appear to play a pivotal role in the early steps necessary for virus entry into cells and, as a consequence, are regarded as the primary targets of a putative protective immune response. The *env* gene shows sequence diversities among different FIV isolates that cluster in discrete segments known as hypervariable (V) regions. Eight or possibly nine such V regions have been identified (Phillips *et al.,* 1990; Morikawa *et al.,* 1991; Rigby *et al.,* 1993; Pancino *et al.,* 1993).

The interactions between FIV and susceptible cells are poorly understood. Similar to HIV, FIV infects T lymphocytes and FIV-infected cats show a marked reduction of circulating CD4$^+$ T lymphocytes (Ackley *et al.,* 1990b; Brown *et al.,* 1991); cells of the monocyte–macrophage–microglia lineage and astrocytes are also infected *in vitro* (Brunner and Pedersen, 1989; Dow *et al.,* 1992; Pedersen, 1993). 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 (Brown *et al.,* 1991). Furthermore, fCD4 expression is restricted to a subpopulation of T lymphocytes and its thymic precursor, and it is unlikely that the fCD4 molecule alone could account for the relatively broad cell tropism of FIV (Ackley *et al.,* 1990a). Recently, a putative non-CD4 receptor has been identified by means of a cell-specific monoclonal antibody, named vpg15, that efficiently blocks FIV infection of susceptible cells *in vitro* but is unable to bind the virus (Hosie *et al.,* 1993). This antibody recognizes a 24-kDa cell-surface protein on feline cells identified as the feline homolog of human CD9, a marker expressed on a range of cell types of both hematopoietic and nonhematopoietic origin (Willet *et al.,* 1994); it has yet to be established, however, whether feline CD9 is a primary binding receptor for FIV or a molecule necessary for a subsequent step in viral entry (Willett and Neil, 1995). On the other hand, no information is available on the SU and/or TM

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domains involved in FIV binding to target cells nor on the other physiological events underlying virus attachment and penetration.

A suitable system for studying *in vitro* interactions between FIV Env domains and target cell molecules is represented by the Crandell feline kidney (CrFK) fibroblastoid cell line and the Petaluma isolate of FIV (FIV-Pet). Four to 6 days after infection with FIV-Pet, these cells develop large syncytia and produce infectious virus detectable as p25 antigen in the culture supernatant (Tozzini *et al.,* 1992, 1993; Lombardi *et al.,* 1994). In this study we have synthesized sixty-six 20- to 23-amino-acid peptides, partially overlapping by 10–12 amino acids and spanning the entire deduced sequences of the SU and TM proteins of FIV-Pet, and examined their ability to inhibit FIV-induced syncytium formation and p25 production. This has led to the identification of two linear domains located, respectively, on the SU and TM envelope glycoproteins that markedly inhibit cell infection by FIV *in vitro.*

MATERIALS AND METHODS

Viruses

Two different FIV isolates, i.e., the California isolate Petaluma (FIV-Pet), kindly provided by Dr. J. Yamamoto (Yamamoto *et al.,* 1988, 1991), and the local isolate Pisa-M2 (FIV-M2) (Baldinotti *et al.,* 1994), have been used. The FIV-M2 isolate was originally obtained from a clinically healthy feline leukemia virus (FeLV)-negative cat and freed of the feline syncytium-forming spumavirus (FeSFV), which was initially present. Unless otherwise stated, viruses were propagated in CrFK cells as previously described (Tozzini *et al.,* 1992). In experiments employing MBM cells (see below), a fresh FIV-M2 isolate was propagated in and harvested from MBM cells, as previously described (Baldinotti *et al.,* 1994). In some experiments, a local isolate of FeSFV, identified by neutralization and immunofluorescence assays with sera kindly provided by Dr. N. C. Pedersen and propagated in CrFK cells, was also used.

Cell lines and culture conditions

CrFK cells were grown in Eagle's minimal essential medium supplemented with 0.5% fetal bovine serum (FBS) and additional supplement (Tozzini *et al.,* 1992). The human erythroleukemia K562 cells, human histiocytic lymphoma U-937 cells, and the interleukin 2- and concanavalin A-dependent, CD4-negative feline T-cell line MBM, established from the peripheral blood mononuclear cells (PBMC) of an FIV- and FeLV-negative SPF cat (Matteucci *et al.,* 1995), were maintained as suspension cultures in RPMI 1640 medium supplemented with 10% FBS, 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Murine 3T3 fibroblasts and primary fibroblasts from SPF cat skin biopsy (275 cells) were maintained as monolayer cultures in Dulbecco's modified minimum essential medium supplemented as above.

Synthetic peptides

On the basis of the published sequence of the 34TF10 clone of FIV-Pet (Talbot *et al.,* 1989), sixty-six 20- to 23-amino-acid peptides, partially overlapping by 10–12 amino acids (see Table 1) and covering the entire SU and TM proteins of the FIV *env* region, were manually synthesized using *N*-α-fluorenylmethoxycarbonyl (FMOC)-protected amino acids and a *p*-alkoxybenzyl alcohol resin as solid phase, as previously described (Lombardi *et al.,* 1993).

Peptides 5, 7, and 59 were also 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 (Tozzini *et al.,* 1992). Briefly, solutions of peptides were added at the final concentration of 16 μg/ml (unless otherwise stated) to 2×10^4 CrFK cells in 0.8 ml of culture medium containing 0.5% FBS in 24-well plates; unless otherwise stated, after 1 hr at 37°C approximately 50–100 syncytium-forming units (SFU) of FIV-Pet or, in some experiments, FIV-M2 or FeSFV were added in 0.2 ml volume. Six days later, the cultures were stained and the number of syncytia was counted under the microscope.

Capture ELISA for p25 antigen

FIV p25 core antigen was assayed by capture ELISA as previously described (Lombardi *et al.,* 1994). Briefly, plates (Probind, Falcon, Italy) were coated overnight with 0.5 μg purified AE11 MAb in 100 μl carbonate buffer, pH 9.6. After four washes with PBS containing 0.05% Tween 20 (PBS–Tw), plates were postcoated 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 four 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) was added and incubated for 1 hr. The plates were then washed and further incubated for 1 hr with 100 μl of a horseradish peroxidase (HPRO)-conjugated anti-biotin (Sigma, St. Louis, MO) diluted 1:1000 in PBS–Tw–BSA. The enzyme reaction was carried out with 100 μl tetramethylbenzidine

TABLE 1

Amino Acid Sequence of the FIV Env Synthetic Peptides Used

Peptide No.	Sequence[a]	Peptide No.	Sequence
1	^{181}PPLVVPVEESEIIFWDCWAPEE^{202}	34	^{517}FNMTKAVEMVNIAGNWSCTS^{536}
2	^{193}IFWDCWAPEEPACQDFLGAMIH^{214}	35	^{527}NIAGNWSCTSDLPSSWGYMN^{546}
3	^{203}PACQDFLGAMIHLKAKTNISIR^{224}	36	^{534}CTSDLPSSWGYMNCNCTNSSSS^{555}
4	^{215}LKAKTNISIREGPTLGNWAR^{234}	37	^{547}CNCTNSSSSYSGTKMACPSNRG^{568}
5	^{225}EGPTLGNWAREIWATLFKKA^{244}	38	^{557}SGTKMACPSNRGILRNWYNP^{576}
6	^{235}EIWATLFKKATRQCRRGRIW^{254}	39	^{567}RGILRNWYNPFAGLRQSLEQ^{586}
7	^{245}TRQCRRGRIWKRWNETITGP^{264}	40	^{577}VAGLRQSLEQYQVVKQPDYL^{596}
8	^{255}KRWNETITGPSGCANNTCYN^{274}	41	^{587}YQVVKQPDYLLVPEEVMEYK^{606}
9	^{265}SGCANNTCYNVSVIVPDYQC^{284}	42	^{593}PDYLLVPEEVMEYKPRRKRAAI^{614}
10	^{275}VSVIVPDYQCYLDRVDTWLQ^{294}	43	^{605}YKPRRKRAAIHVMLALATVLSI^{626}
11	^{285}YLDRVDTWLQGKINISLCLT^{304}	44	^{615}HVMLALATVLSIAGAGTGATAI^{636}
12	^{295}GKINISLCLTGGKMLYNKVT^{314}	45	^{627}AGAGTGATAIGMVTQYHQVL^{646}
13	^{305}GGKMLYNKVTKQLSYCTDPL^{324}	46	^{637}GMVTQYHQVLATHQEAIEKV^{656}
14	^{315}KQLSYCTDPLQIPLINYTFG^{334}	47	^{647}ATHQEAIEKVTGALKINNLR^{666}
15	^{325}QIPLINYTFGPNQTCMWNTS^{344}	48	^{657}TGALKINNLRLVTLEHQVLV^{676}
16	^{335}PNQTCMWNTSQIQDPEIPKC^{354}	49	^{667}LVTLEHQVLVIGLKVEAMEK^{686}
17	^{345}QIQDPEIPKCGWWNQMAYYN^{364}	50	^{677}IGLKVEAMEKFLYTAFAMQE^{696}
18	^{355}GWWNQMAYYNSCKWEEAKVK^{374}	51	^{687}FLYTAFAMQELGCNQNQFFC^{706}
19	^{366}CKWEEAKVKFHCQRTQSQPGSW^{387}	52	^{697}LGCNQNQFFCKIPLELWTRY^{716}
20	^{375}FHCQRTQSQPGSWFRAISSWKQ^{396}	53	^{707}KIPLELWTRYNMTINQTIWN^{726}
21	^{385}GSWFRAISSWKQRNRWEWRPDF^{406}	54	^{717}NMTINQTIWNHGNITLGEWY^{736}
22	^{395}KQRNRWEWRPDFKSKKVKISLPC^{417}	55	^{727}HGNITLGEWYNQTKDLQQKF^{746}
23	^{407}KSKKVKISLPCNSTKNLTFA^{426}	56	^{737}NQTKDLQQKFYEIIMDIEQN^{756}
24	^{417}CNSTKNLTFAMRSSGDYGEV^{436}	57	^{747}YEIIMDIEQNNVQGKTGIQQ^{766}
25	^{427}MRSSGDYGEVTGAWIEFGCH^{446}	58	^{757}NVQGKTGIQQLQKWEDWVRW^{776}
26	^{437}TGAWIEFGCHRNKSNLHTEA^{456}	59	^{767}LQKWEDWVRWIGNIPQYLKG^{786}
27	^{447}RNKSNLHTEARFRIRCRWNV^{466}	60	^{777}IGNIPQYLKGLLGGILGIGL^{796}
28	^{457}RFRIRCRWNVGSDTSLIDTC^{476}	61	^{787}LLGGILGIGLGVLLLILCLP^{806}
29	^{467}GSDTSLIDTCGNTPNVSGAN^{486}	62	^{797}GVLLLILCLPTLVDCIRNCI^{816}
30	^{477}GNTPNVSGANPVDCTMYSNK^{496}	63	^{807}TLVDCIRNCIHKILGYTVIA^{826}
31	^{487}PVDCTMYSNKMYNCSLQNGF^{506}	64	^{817}HKILGYTVIAMPEVEGEEIQ^{836}
32	^{497}MYNCSLQNGFTMKVDDLIVH^{516}	65	^{827}MPEVEGEEIQPQMELRRNGR^{846}
33	^{507}TMKVDDLIVHFNMTKAVEMV^{526}	66	^{837}PQMELRRNGRQCGMSEKEEE^{856}

[a] Deduced from the 34TF10 clone of FIV-Pet (Talbot *et al.,* 1989).

(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 RT. Duplicate wells containing twofold dilutions of recombinant p25 (kindly provided by Dr. O. Jarrett) ranging from 500 to 0.03 ng/ml served for standard curve.

FITC-labeling of peptides

One milliliter of peptide solutions in 0.1 *M* Na_2CO_3, pH 9.0 (1.5 mg/ml), was mixed with 50 μl of fluorescein isothiocyanate (FITC, Sigma) diluted at 1 mg/ml in dimethyl sulfoxide and incubated overnight at 4°C. NH_4Cl (50 m*M* final concentration) was added and further incubated at 4°C for 2 hr. The peptides were then freed of unbound FITC by passing through a Sephadex G-25 column.

Flow cytometry

Cells (2×10^5), washed twice with PBS containing 1% BSA, were incubated for 30 min at 4°C with fluorescein-conjugated peptide at the final concentration of 10 μg/ml in a 100-μl volume of PBS containing 1% BSA. After two further washings, cells were fixed in PBS containing 1% *p*-formaldehyde, 1% BSA, and 0.1% sodium azide. Flow cytometry analysis was performed with an Epics Elite cell analyzer (Coulter Electronics, Hialeah, FL) equipped with an air-cooled argon laser operating at 15 mW and 488-nm line emission. Viable cells were gated in a two-parameter histogram of size versus granularity and approximately 10,000 cells were collected in each fluorescence histogram, as previously described (Lombardi *et al.,* 1994).

Circular dichroism (CD) measurements

CD spectra were recorded at 25°C on a Jasco J500A spectropolarimeter in 10 m*M* sodium phosphate buffer, pH 7.0l, containing 150 m*M* NaCl or in the same buffer in the presence of 90% methanol (v/v) or 40 m*M* sodium dodecyl sulfate (SDS). Data are expressed as molar ellip-

ticity [Θ] (deg cm^2 dmol^{-1}), based on the mean residue molecular weight.

RESULTS

Screening of Env peptides for inhibition of FIV-induced syncytium formation

Previous studies have shown that inhibition of syncytium formation in CrFK cells is a useful screening method for detecting substances that block FIV infectivity, such as broadly reactive neutralizing antibodies (Tozzini *et al.,* 1993). In a first group of experiments the 66 partially overlapping synthetic peptides covering the entire Env glycoproteins of FIV were screened for the capacity to affect FIV replication by using this approach. To this purpose, 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 were counted 6 days later. Figure 1 summarizes the results from numerous experiments in which each peptide was tested at least three times. In spite of a certain degree of variation in different experiments, the peptides corresponding to two specific domains of the Env proteins exhibited a remarkable and highly reproducible antiviral effect, reducing syncytium formation by over 90% in all the experiments. One such domain, represented by peptides 5, 6, and 7, is located in the conserved near-amino-terminal portion of the SU protein spanning amino acids ^{225}E–P^{264}. The second domain, represented by peptides 58, 59, 60, and 61, is located in the external portion of the TM protein adjoining the transmembrane region, spanning amino acids ^{757}N–P^{806}, and encompasses a segment that presents a considerable degree of sequence variation, known as hypervariable region 8 (Phillips *et al.,* 1990; Morikawa *et al.,* 1991; Rigby *et al.,* 1993; Pancino *et al.,* 1993). Peptides derived from a third region, located around the cleavage site of the SU and TM proteins, were also inhibitory (peptides 38 to 50), but the reduction of syncytium formation produced seldom exceeded 60%. Inhibition levels lower than 30% were not considered of interest. Peptide 19, spanning amino acids ^{367}C–W^{387} and located in FIV V3 loop, exerted an enhancing effect on syncytium production (unpublished observations). A similar enhancement of infection has been reported for HIV-1 V3 loop synthetic peptides (Zanotto *et al.,* 1995).

Although we cannot exclude that the assay we used may miss some inhibitory peptide, on the basis of the findings reported above, we selected peptides 5, 7, 58, and 59 for further studies. Peptide 66 and occasionally peptides 36 and 54 were used as a noninhibitory negative controls.

Inhibition of FIV replication in CrFK and MBM cells by selected Env peptides

The peptides that had proven potent inhibitors of FIV-induced syncytium formation were examined directly for the ability to block FIV replication by measuring the amount of p25 released in the supernatant fluids of infected cultures. They were first tested against FIV-Pet in CrFK cells. As shown in the experiment depicted in Fig. 2, peptides 5, 7, 58, and 59 reduced syncytium formation and p25 antigen production in a dose-dependent manner. The lowest concentrations of peptide required to reduce by 50% the amount of p25 antigen produced ranged between 1 μg/ml (peptides 58 and 59) and 8 μg/

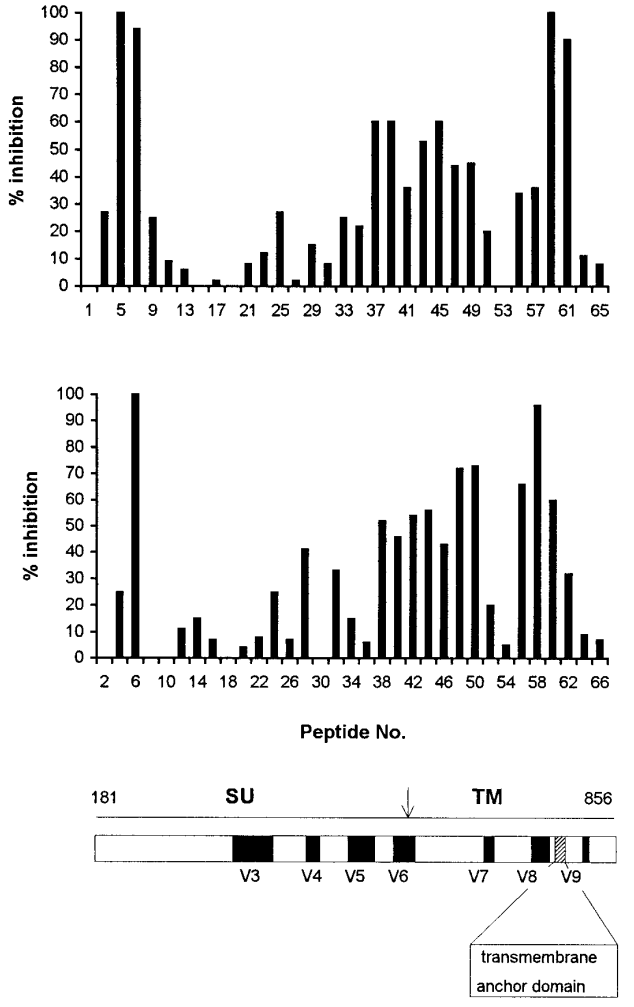

FIG. 1. Inhibition of FIV-induced syncytium formation by 66 synthetic peptides covering the entire SU and TM proteins of FIV. Peptides at 16 μg/ml (final concentration) were incubated for 1 hr with CrFK cells that were then infected with 50–100 SFU of FIV-Pet adapted to grow in these cells. Syncytia were counted 6 days later. The inhibition values reported represent the arithmetic means of the inhibition values observed for each peptide in at least three independent experiments. The location of the synthetic peptides on the Env sequence is indicated along the *x*-axis together with the location of hypervariable (V) regions. Arrow indicates the splicing site between surface (SU) and transmembrane (TM) glycoproteins; numbers above the continuos horizontal line show the amino acid positions in the *env* gene product. As the peptides overlapped one another in such a way as to reproduce the Env proteins twice, the results obtained with the even and odd peptides are shown in separate panels to better locate peptide activities on the Env protein sequences.

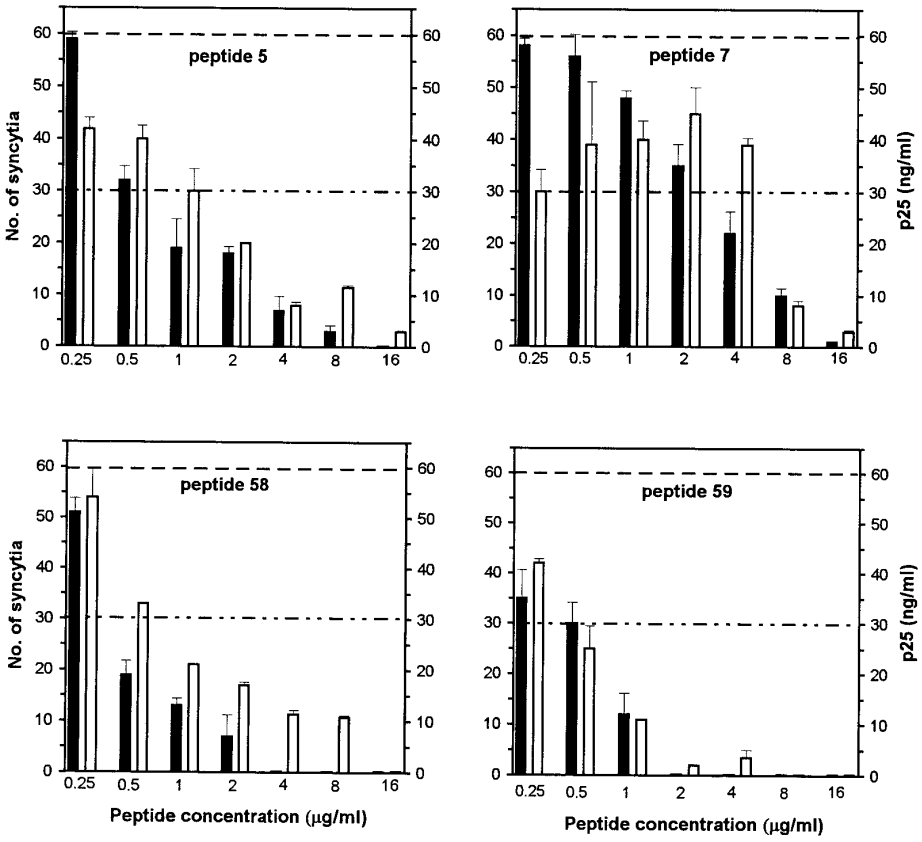

FIG. 2. Dose dependence of inhibition of FIV-induced syncytium formation and p25 production by selected Env peptides in CrFK cells. Peptides were incubated at final concentrations ranging from 0.25 to 16 μg/ml for 1 hr with CrFK cells that were then infected with 50–100 SFU of FIV-Pet adapted to grow in these cells. Syncytia (■) and p25 levels (□) were counted 6 days later; bars represent the mean ± 1 SD of duplicate cultures (some error bars are smaller than the markers and do not appear in the figure). Horizontal dashed and dotted–dashed lines represent the mean numbers of syncytia and p25 levels, respectively, in the absence of peptide of triplicate cultures; SD were 3.2 and 2.7, respectively (not shown). Peptide 66 (negative control) did not affect the numbers of syncytia and p25 level at all tested doses (data not shown).

ml (peptide 7). In the experiment shown in Fig. 2 the peptides also determined an increase in p25 levels at low concentrations; the magnitude of this effect, however, varied considerably in different experiments.

Additional studies performed with the local isolate FIV-M2 adapted to CrFK cells gave similar overall results, thus showing that the blocking activity of the peptides is not restricted to the homologous viral isolate after which the peptides were modeled (namely FIV-Pet), but extended to a heterologous virus (data not shown; see also Table 3). FIV-Pet and FIV-M2 show considerable genetic diversity and have been classified in different clades (clades A and B, respectively) based on partial *gag* and *env* gene sequences (Cammarota *et al.,* 1996).

The FIV isolates used in the previous experiments were laboratory strains adapted to grow in CrFK cells and had been passaged *in vitro* 234 (FIV-Pet) and 18 times (FIV-M2). Since prolonged growth *in vitro* has been shown to significantly modify several properties of lentiviruses including sensitivity to blocking agents such as neutralizing antibody and soluble receptor (Baldinotti *et al.,* 1994; Golding *et al.,* 1994; Moore *et al.,* 1995), we tested peptides 5, 7, 58, and 59 for the ability to inhibit a fresh isolate of FIV. The virus used was an early passage of FIV-M2 that had been propagated *in vitro* only 6 times and solely in nontransformed lymphoid cells, and the cell type used as substrate was the T lymphocyte cell line MBM. Previous work has shown that the target cell used may also affect the results of FIV blocking experiments (Baldinotti *et al.,* 1994). MBM cells were cultured at 5×10^4 cells per well in 96-well microplates in the presence of varying concentrations of the peptides and, after 1 hr at 37°C, 10 $TCID_{50}$ of FIV-M2 were added to each well. The amounts of p25 antigen released in the culture supernatants were measured 4, 8, and 12 days later. Each peptide was tested at least twice in quadruplicate. The results showed that SU peptides 5 and 7 were also active in this infection system, though less efficiently than in the CrFK system. At concentrations of 1 and 2.5 μg/ml they had no detectable effects on FIV-M2 replication and at 5 μg/ml inhibited virus replication by approximately 50% and with a certain variability in different experiments (data not shown). At 10 μg/ml (see Table 2), these peptides significantly inhibited FIV repli-

TABLE 2

Effect of Selected Env Peptides on FIV-M2 Replication in MBM Cells

Exp No.	Peptide[a]	Day of culture 4	8	12
1	None	18 ± 2[b]	51 ± 18	>250
	54 (−ctrl)	20 ± 1	75 ± 9	>250
	36 (−ctrl)	20 ± 3	60 ± 14	>250
	5	15 ± 1*	26 ± 8*	245 ± 10
	7	13 ± 1*	15 ± 7*	154 ± 65*
	59	19 ± 1	59 ± 14	>250
2	None	23 ± 1	54 ± 16	246 ± 5
	66 (−ctrl)	24 ± 1	ND	184 ± 105
	5	21 ± 3	36 ± 15	62 ± 34*
	7	15 ± 1*	6 ± 3*	32 ± 14*
	58	21 ± 2	37 ± 14	230 ± 28

Note. ND, not determined.

[a] Peptides were tested at the final concentration of 10 μg/ml.

[b] Data expressed as mean ± SD ng/ml of p25 in quadruplicate culture supernatants.

* $P < 0.05$, according to Student's *t* test, when compared to control cultures in the absence of peptide.

cation although with some scatter in different experiments. In contrast, peptides 58 and 59 failed to demonstrate an antiviral effect. As expected, negative control peptides 36, 54, and 66 exhibited no inhibitory activity.

Specificity of the anti-FIV activity of Env peptides

At the concentrations used, the FIV-inhibitory peptides exerted no overt effects on CrFK or MBM cell growth or morphology, thus excluding that inhibition of FIV replication was a mere consequence of peptide cytotoxicity or cytostasis. To exclude that the inhibitory effects were due to impurities or unwanted peptides present in the synthetic peptide preparations, new batches of peptides 5, 7, and 59 were synthesized and highly purified, and the identity of the final products was verified by electrospray mass spectrometry. The newly synthesized peptides inhibited FIV replication and syncytium formation as effectively as the original batches (data not shown). In additional experiments, peptides 5, 7, 58, 59, and 66 (negative control) were examined for the ability to affect syncytium production in CrFK cells by an unrelated feline retrovirus, FeSFV. Table 3 shows the results of one representative experiment. In the presence of the FIV-inhibitory peptides added to the cultures 1 hr prior to virus inoculation, FeSFV produced numbers of syncytia similar to those produced in the absence of peptides or in the presence of negative control peptides. Consistent with previous results, in the same experiment FIV-Pet and FIV-M2 underwent the expected degrees of peptide-mediated inhibition (Table 3).

Effect of varying the timing of FIV-inhibitory peptide administration

In these experiments, CrFK cells grown in 24-well plates were mixed with approximately 100 SFU of FIV-Pet and 1, 3, 8, and 24 hr later treated with peptides 5, 7, 58, or 59. Positive controls consisted of cultures receiving peptides 1 hr before virus; negative control cultures received no peptides or peptide 66 at all tested times. After 6 days incubation, 2 wells for each peptide/timing combination were examined for FIV-induced syncytia and p25 content in the supernatant fluids. As shown in Fig. 3, peptides 5, 7, 58, and 59, as expected, determined a marked inhibition of both syncytium formation and p25 release when added to the cultures 1 hr before virus infection. Moreover, the addition of peptide 59 exerted the most powerful inhibitory effect, virtually preventing syncytium formation and p25 antigen production at all the times tested after FIV infection within the 24 hr studied. FIV inhibition by peptide 58 was also affected little by varying the time of posttreatment and was visible on both syncytium formation and p25 antigen production but was much less pronounced than in the experiments in which the same peptide was added before the cells were exposed to the virus. In contrast, peptides 5 and 7 seem more time-dependent in their action, producing a clear-cut reduction of the numbers of syncytia produced only when added within 1 and 3 hr after virus inoculation, respectively; on the other hand, the levels of p25 produced were appreciably reduced only by peptide 7 added within 3 hr postinfection. FIV absorption to CrFK cells can be considered complete by this time (Tozzini *et al.,* 1992). When peptides were added 8 or 24 hr postinfection we observed a slight reduction of numbers of syncytia, but the levels of p25 were not significantly affected.

Binding of FIV-inhibitory Env peptides to substrate cells

To investigate whether inhibition of FIV replication correlated with Env peptide binding to substrate cells, CrFK and MBM cells were examined for the ability to bind peptides 5, 7, 58, 59, and 66 (negative control). As judged from the proportion of fluorescent cells observed by flow cytometry following incubation with FITC-labeled peptides at 4°C for 30 min, peptides 5, 7, and 59 were bound effectively by both CrFK and MBM cells while peptide 58 was bound by CrFK cells only and peptide 66 was not bound by either cell type. Interestingly, a similar binding pattern was observed with two other cell types that are permissive for FIV replication and express the putative FIV receptor recognized by monoclonal antibody vpg15, namely fresh feline PBMC and primary feline lymphoblasts obtained by stimulating PBMC with Con A for 3 days (PBMC–Con A), whereas several FIV-nonpermissive cells failed to bind the peptides. The latter cells included primary feline fibroblasts that expressed the vpg15 antigen but did not replicate FIV to any detectable

TABLE 3

Effect of Selected Env Peptides on FIV and FeSFV Infection of Feline CrFK

Peptide	FIV-Pet		FIV-M2		FeSFV
	No. of syncytia	p25 (ng/ml)	No. of syncytia	p25 (ng/ml)	No. of syncytia
None	57 ± 4	85 ± 6	28 ± 2	350 ± 45	118 ± 3
66 (−ctrl)	54 ± 4	78 ± 7	23 ± 7	300 ± 30	124 ± 12
5	1 ± 1	0	2 ± 1	20 ± 10	109 ± 14
7	4 ± 3	5 ± 2	1 ± 1	62 ± 10	104 ± 7
58	0	9 ± 1	0	103 ± 25	101 ± 8
59	0	0	0	0	111 ± 10
61	2 ± 1	14 ± 7	0	38 ± 3	112 ± 11

Note. CrFK cells were infected with approximately 50, 25, and 100 SFU of FIV-Pet, FIV-M2, and FeSFV, respectively. Numbers of syncytia and levels of p25 produced were evaluated after 6 days of culture and expressed as mean ± SD of triplicate cultures.

extent (unpublished observation), as well as the murine cell line 3T3, fresh human PBMC, and the human cell line K562, which were all vpg15 negative. However, the human cell line U937, which is also vpg15 negative, bound peptides 7 and 59 at least as effectively as CrFK cells (Table 4).

CD analysis of selected peptides

Peptides 5, 7, 58, and 59 were analyzed by circular dichroism under various solution conditions. CD spectra of peptides 7, 58, and 59 indicate that the peptides adopt

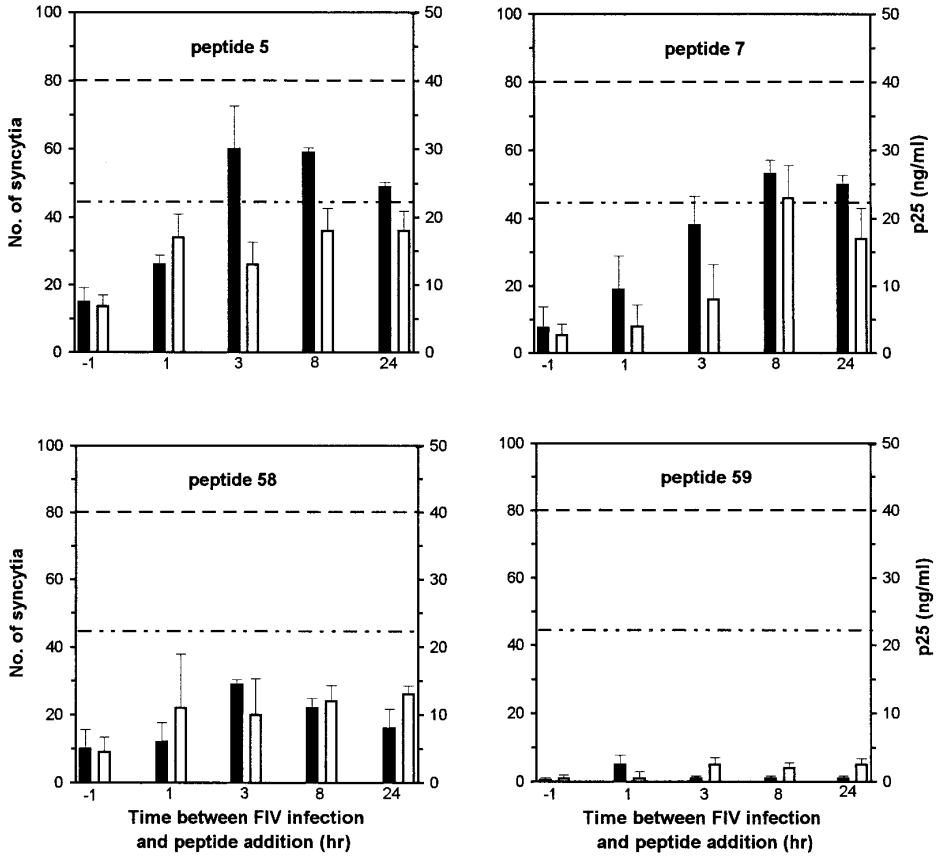

FIG. 3. FIV-induced syncytium formation and p25 production in CrFK cells treated with selected Env peptides at various times after FIV infection. CrFK cells were infected with approximately 100 SFU of FIV-Pet adapted to grow in these cells and 1, 3, 8, and 24 hr later treated with peptides at the final concentration of 16 μg/ml. Inhibition control cultures received peptides 1 hr before virus (time = −1). Syncytia (■) and p25 levels (□) were counted 6 days later; bars represent the mean ± 1 SD of duplicate cultures. Horizontal dashed and dotted–dashed lines represent the mean numbers of syncytia and p25 levels, respectively, in the absence of peptide of triplicate cultures; SD were 1.7 and 2.5, respectively (not shown). Peptide 66 (negative control) did not affect the number of syncytia and p25 levels at all tested times (data not shown).

TABLE 4

Binding of Selected Env Peptides to Different Cell Types

Cells	Type	Percentage of positive cells[a]					
		Peptide					
		66 (−ctrl)	5	7	58	59	Antibody vpg15
CrFK	Cat fibrolastoid kidney line	1.5	32	49	26	30	100
MBM	Cat T-cell line	0.8	39	43	0.0	35	100
fPBL	Primary cat PBMC	1.5	29	10	4	14	50
fPBL-Con A	Con A-stimulated primary cat PBMC	1.2	25	5	3	21	45
275	Primary cat fibroblasts	0.3	0.2	0.3	0.1	0.1	100
hPBMC	Primary human PBMC	0.0	0.0	0.0	0.0	0.0	ND
3T3	Mouse fibroblast line	0.1	0.6	0.0	0.3	0.5	0.5
U937	Human histiocytic lymphoma cell line	0.2	0.2	49	0.1	41	0.0
K562	Human chronic myeloid leukemia line	0.1	0.2	0.1	0.1	0.0	0.0

[a] Assessed by flow cytometry using FITC-labeled peptides.

random coil structure under all the conditions studied (not shown). On the other hand, in 10 m*M* sodium phosphate buffer, pH 7.0l, containing 150 m*M* NaCl, the spectrum of peptide 5 showed a weak band at about 220 nm and a stronger negative band near 200 nm (Fig. 4, curve 1), thus indicating that under these conditions peptide 5 also adopts an essentially random coil structure, as usually expected for short peptides. The weak band at 220 nm suggests the presence of a very small amount of helical structure. The CD spectrum of peptide 5 changed markedly in the presence of SDS, at concentrations above its critical micelle concentration, or methanol (curves 2 and 3, respectively). Under these conditions the spectra exhibited the two negative minima at 222 and 208 nm, typical of the α-helix structure. Assuming $[\Theta]_{222}$ = ca. −30,000 deg cm^2 dmol^{-1} for a 100% helical structure containing 19 peptide chromophores at 25°C (Benson *et al.,* 1995; Scholtz *et al.,* 1991), 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 seven residues of the peptide sequence form two α-helix turns.

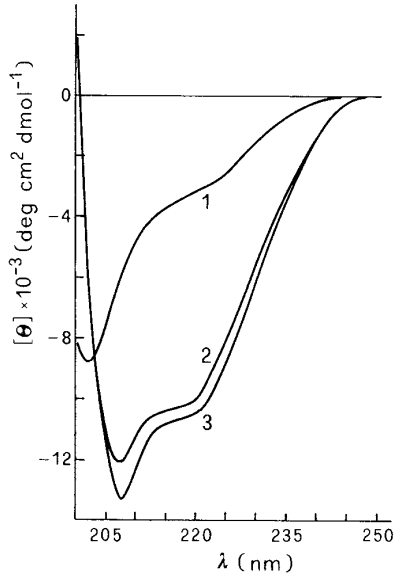

FIG. 4. Circular dichroism spectra of peptide 5 in 10 m*M* sodium phosphate buffer, pH 7.0l, containing 150 m*M* NaCl (curve 1) or in the same buffer in the presence of 40 m*M* SDS (curve 2) or 90% methanol (v/v) (curve 3). The spectra were found to be independent of peptide concentration in the range of 8×10^{-2} to 8×10^{-1} g/liter, thus showing that the peptide molecules are monomeric and do not aggregate in solution.

DISCUSSION

In this first report investigating the effects of Env synthetic peptides on FIV replication, peptides derived from two regions of the *env* gene product of FIV-Pet were found to exert a remarkable inhibitory action on CrFK cell infection with the homologous and a heterologous FIV isolate *in vitro.* The amino acid sequences involved span residues ^{225}E–P^{264} of the SU glycoprotein (peptides 5 through 7) and residues ^{757}N–P^{806} of the TM molecule (peptides 58 through 61). The inhibitions are apparently specific for FIV, as the peptides proved unable to inhibit infection of the same cell substrate with a different syncytium-inducing retrovirus, i.e., FeSFV. The SU peptides, but not the TM peptides, also inhibited infection of lymphoid MBM cells by a fresh FIV isolate.

Antiviral activity of synthetic peptides deduced from Env glycoproteins has also been reported for HIV-1, in which inhibitory domains have been mapped in the V3 principal neutralizing domain loop of gp120 and in the ectodomain of gp41. In spite of structural and immunological similarities between the V3 regions of HIV-1 and FIV (Lombardi *et al.,* 1993), the peptides deduced from

the FIV V3 loop, i.e., peptides 19 through 22, showed no blocking activity, as well as no sequence homology with the inhibitory peptides of HIV-1 V3. The inhibitory peptides of FIV SU glycoprotein encompass a sequence that is distant from V3 and that, according to an FIV Env glycoprotein structural model recently proposed (Pancino *et al.,* 1993), contains an α-helix structure. Moreover, this sequence is conserved among different FIV isolates and seems not to be exposed on the surface of the SU molecule as it forms a groove in the context of glycoprotein internal folding (Pancino *et al.,* 1993).

The TM inhibitory sequence, corresponding to peptides 58–61, is located in the external portion of the TM glycoprotein adjoining the transmembrane region and encloses, particularly with peptides 58 and 59, the whole domain V8, i.e., one of the three hypervariable regions of the TM glycoprotein (Pancino *et al.,* 1993). It is noteworthy that in HIV-1 two partially overlapping synthetic peptides, named DP178 (Wild *et al.,* 1993, 1994) and SJ2176 (Neurath *et al.,* 1995), which inhibit HIV-1 replication *in vitro,* are also localized near the membrane-spanning domain of the gp41 glycoprotein. This could suggest that a domain of the external portion of the TM glycoproteins of lentiviruses, next to the viral envelope membrane, plays an important role in cell infection. It is usually accepted that, irrespective of primary sequence, a common general framework exists in the *env* gene products of different lentiviruses (Gallaher *et al.,* 1989, 1995; Schulz *et al.,* 1992; Pancino *et al.,* 1993, 1994).

At this time, it is difficult to envision the mechanism(s) by which SU and TM synthetic peptides block FIV replication. In HIV-1, several different mechanisms have been proposed to explain the inhibitory action of Env peptides; however, it is generally agreed that both gp41 and gp120 peptides interfere with cell infection at early steps necessary for virus entry into host cells (Wild *et al.,* 1994; Jiang *et al.,* 1993a,b; Yahi *et al.,* 1995a). In particular, it has been reported that synthetic peptides of HIV-1 gp120 block cell infection by directly interacting with cell-surface molecules; in fact, peptides or polymeric constructs derived from V3 bind CD4, the major HIV-1 receptor, and the non-CD4 receptor galactosylceramide and other potential coreceptors (Benjouad *et al.,* 1995; Yahi *et al.,* 1994, 1995a,b). In the FIV model, SU peptides 5 and 7 bound the surface of FIV permissive cells and were highly active in blocking FIV infection when administered before the virus, but were not active when added to cells a few hours after initial contact with the virus. This might suggest that the SU peptides specifically interact with cell-surface molecules involved in viral infection and compete with FIV binding to cell receptor(s). Moreover, CD analysis showed that peptide 5 is essentially random coil under physiological conditions, but undergoes a marked conformational change and shows a significant increase of the α-helix conformation upon going to more hydrophobic environments, such as SDS micelles. This may suggest that in proximity to cellular membrane, where an apolar environment exists, peptide 5 may assume a more stable secondary structure that might account for its antiviral and cell-surface binding activities. In HIV-1, the inhibitory synthetic peptide DP107 exhibits, under physiological conditions, an α-helix conformation characterized by a "leucine zipper-like motif," a domain probably involved in the infection event (Wild *et al.,* 1992; Bernstein *et al.,* 1995). Thus, the fact that peptides endowed with an α-helix motif, although differently located in the envelope glycoproteins of FIV and HIV-1, are able to inhibit cell infection may indicate that the antiviral activity of certain retroviral Env peptides depends on their ability to accurately model functional viral protein domains.

The mechanism(s) whereby TM peptides 58 and 59 block FIV cell infection is not understood. In HIV-1, the inhibitory peptides of gp41 seem to exert their antiviral activity by interacting with other regions of the gp41 molecule itself; in fact, a peptide of the gp41 ectodomain, coded DP-178 (Wild *et al.,* 1995), interacts with the α-helical structure exhibited by the above-mentioned peptide DP-107 (Wild *et al.,* 1992), thus causing the disruption of the α-helical leucine zipper-like motif and inhibiting HIV-1 cell infection. Another HIV-1 gp41 peptide, indicated as SJ2176 (Jiang *et al.,* 1993a,b; Neurath *et al.,* 1995) and overlapping DP-178 (Neurath, *et al.,* 1995), shows selective binding to the N-terminal viral fusion domain of gp41 (Jiang *et al.,* 1993b; Neurath *et al.,* 1995); this peptide seems to inhibit HIV-1 infection by preventing the interaction between the fusogenic domain and relevant cell membrane constituents. Nevertheless, it has also been reported that another HIV-1 gp41 inhibitory peptide, named CS3, blocks HIV entry into human $CD4^+$ cells by directly binding to two proteins of the cell surface, respectively, of 45 and 80 kDa (Henderson and Qureshi, 1993). In FIV, TM peptides 58 and 59, though capable of binding FIV permissive cells, inhibit FIV infection also when added after the virus. This might indicate that TM peptides interfere with virus replication at a step subsequent to virus absorption. Moreover, our data suggest that FIV inhibition by these peptides might be dependent on cell type and/or viral isolate. In CrFK cells they exerted an antiviral activity against homologous and heterologous FIV isolates and gave 100% reduction of syncytium formation at a concentration 10 times lower than SU peptides but, contrary to SU peptides, were inactive when tested in lymphoid MBM cells infected with a fresh FIV isolate.

Finally, a common feature of SU and TM inhibitory peptides of FIV is the lack of antigenic activity; we have in fact observed that synthetic peptides 5, 7, 58, and 59 are not recognized by sera of cats infected with FIV-Pet or FIV-M2 and do not induce detectable humoral responses when repeatedly injected into mice (Massi *et al.,* manuscript in preparation). This should prove useful

in future studies, since antibody-mediated clearance will not hinder evaluation of the peptides as anti-FIV agents *in vivo.* Also, it is tempting to speculate that lack of immunogenicity of critical segments of the Env molecules is yet another escape mechanism that protects the virus from a host's immune response.

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