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Retention of Viral Infectivity after Extensive Mutation of the Highly Conserved Immunodominant Domain of the Feline Immunodeficiency Virus Envelope

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In lentiviruses, including human immunodeficiency virus and feline immunodeficiency virus (FIV), the principal immunodominant domain (PID) of the transmembrane glycoprotein elicits a strong humoral response in infected hosts. The PID is marked by the presence of two cysteines that delimit a sequence, composed of five to seven amino acids in different lentiviruses, which is highly conserved among isolates of the same lentiviral species. While the conservation of the sequence suggests the presence of functional constraints, the conservation of the immunodominance among divergent lentiviruses raises the hypothesis of a selective advantage for the infecting virus conferred by the host humoral response against this domain. We and others have previously shown that an appropriate structure of the PID is required for the production of a functional envelope. In the present work, we analyzed virological functions and immune reactivity of the envelope after random mutagenesis of the PID of FIV. We obtained nine mutant envelopes which were correctly processed and retained fusogenic ability. Mutation of the two C-terminal residues of the PID sequence between the cysteines in a molecular clone of FIV abolished infectivity. In contrast, three molecular clones containing extensive muta tions in the four N-terminal amino acids were infectious. However, the mutations affected PID reactivity with sera from infected cats. Our results suggest that functional constraints, although existent, are not sufficient to account for PID sequence conservation. Such conservation may also result from positive selection by anti-PID antibodies which enhance infection.

The envelope glycoproteins of lentiviruses form oligomeric structures at the surfaces of virions and infected cells. These structures are composed of two subunits, the surface glycoprotein (SU) and the transmembrane glycoprotein (TM) (reviewed in reference 13). The envelope glycoproteins display high variability, but the variable positions are not randomly distributed and their distribution is thought to reflect structural constraints and immunological pressures. For example, antigenic variation might be driven by the host neutralizing-antibody response, and high conservation of primary structure is often a hallmark of a region possessing an essential virological function. Interestingly, in the external portion of the TMs of all lentiviruses there is a domain which is highly conserved and strongly immunogenic. This domain contains two proximal cysteines probably linked to form a loop of seven to nine amino acids (9, 19). A strong humoral response against this domain arises during the course of infection and accounts for a substantial portion of the circulating antiviral antibodies. This holds true for all lentivirus-host systems studied so far. Most, if not all, sera from infected individuals or animals are reactive with peptides corresponding to the sequence of this region (2, 4, 8, 10, 17, 23). For these reasons, this domain has been termed the principal immunodominant domain (PID). Despite these unique immunological properties, and in contrast to other antigenic regions of the envelope, the PID, and particularly the sequence contained between the two cysteines, is conserved among different isolates of the same lentiviral species. For example, the sequence CNQNQFFC is conserved among all isolates of feline immunodeficiency virus (FIV). If conservation of the PID among lentiviruses suggests an important functional role for this domain in the viral cycle, the unusual conservation of its immunodominance suggests that selective pressure for conservation could be exerted by a humoral response that would be favorable to the virus.

A previous work from our laboratory has addressed the functional role of the PID structure by site-directed mutagenesis of the FIV PID (21). The disruption of the PID loop structure, such as by mutation of one or both cysteines, caused severe impairment of envelope precursor processing, resulting in nonfunctional envelopes. The kinetics of SU release for some mutant envelopes also supported the previously formulated hypothesis that the PID is involved in association of SU and TM (26–28). Interestingly, the replacement of the FIV sequence between the cysteines with the corresponding sequence of the human immunodeficiency virus type 1 (HIV-1) PID permitted the reconstitution of a fusogenic envelope. However, this PID exchange caused the loss of viral infectivity. Therefore, this study demonstrated that the loop structure of the PID is essential to the production of a functional mature envelope but raised the possibility that modification of its sequence is compatible with the retention of certain envelope functions.

In the present study, in order to determine the relative importance of functional and immunological constraints in the conservation of the PID loop sequence, we performed random mutagenesis of the sequence contained between the cysteines of the PID of the FIV envelope. Nine mutant envelopes displaying a wide range of mutations efficiently mediated syncytium formation, and three of them were able to reconstitute infectious viruses. However, the mutations caused consider-

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able reduction or abolition of PID reactivity with sera from FIV-infected cats. These results indicate that extensive mutation of the PID loop sequence is tolerated without a loss of essential virological functions but causes modification of the antigenicity of the PID. Immunological mechanisms that favor the virus, such as enhancement antibodies, might be associated with structural constraints in exerting a selective pressure for conservation of the PID.

MATERIALS AND METHODS

Random mutagenesis of the FIV PID. The Env expression vector pTΔ20 (22) and the corresponding mutant envelope expression vector M1 (CΔC), which encodes a protein with a deletion of the sequence between the two cysteines of the PID (21), were modified by deleting the existing *Sac*I site at position 504 by *Sac*I digestion and religation after Klenow treatment. The *Sac*I-deleted pTΔ20 clone, pTΔ20ds, did not show any diminution in syncytium-inducing ability compared to pTΔ20 after transfection in CrFK cells.

We performed random mutagenesis of either the four amino acids following the N-terminal cysteine or the last two amino acids before the C-terminal cysteine of the PID loop. The rationale for this choice is the observation that the PID loop sequence of FIV can be considered composed of two motifs with respect to functional and structural properties of amino acids: the N-terminal sequence NQNQ is formed by four polar residues of the amide class, which are expected to have external positions, while the C-terminal FF residues are aromatic, hydrophobic, and internal. Mutagenesis was performed by PCR with mixtures of oligonucleotides varying at defined positions of the PID-encoding sequences (degenerate primers). A *Sac*I site was introduced into the primers at position 8353 (the numbering used is that used for FIV 34TF10 [31]) upstream of the PID loop (8358 to 8381), without changing the encoded amino acids, in order to clone the PCR products into the mutant envelope M1 (CΔC) (see below). PCR was performed for 38 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) in a Perkin-Elmer Gene System 9600, using pTΔ20ds as the template. Two forward primers containing the *Sac*I site (underlined) and degenerate in the codons coding either for the four N-terminal amino acids or for the two C-terminal amino acids of the PID loop (primer 1 [8346 to 8381], 5′-CAAGAGCTCGGAGGATGTNNNNNNNNNNNNTTTTTCTGC-3′; primer 2 [8346 to 8389], 5′-CAAGAGCTCGGAGGATGTAATCAAAATCAANNNNNNTGCAAAATCCC-3′) and a reverse primer containing an *Nde*I site (position 8903) (8901 to 8928, 5′-GATTTGATTCGAAATGGATTCATATGAC-3′) were used to generate a fragment of 582 bp. The PCR products were digested by *Sac*I and *Nde*I, purified by using the Wizard PCR Preps kit (Promega), and cloned into the M1 (CΔC) clone (previously digested with *Sac*I and *Nde*I and purified with Gene Clean to eliminate the *Sac*I-*Nde*I fragment). The mutant M1 (CΔC) envelope, which is not fusogenic (21), was used for cloning the PCR inserts to allow direct screening of recombinant clones via a syncytium formation assay (22). Moreover, the deletion of 18 bp of the sequence encoding the PID in the M1 (CΔC) envelope allowed an initial selection of recombinants based on the modified mobility of the recombinant *Sac*I-*Nde*I fragments on a 1.5% agarose gel. Minipreps corresponding to selected clones were purified by using the Wizard Minipreps DNA purification system, phenol-chloroform extracted, and ethanol precipitated. Recovered DNA was evaluated by restriction enzyme digestion and agarose gel electrophoresis and used to transfect CrFK cells seeded in six-well plates. Mutated domains of all clones that induced syncytium formation and of some clones that did not were sequenced with a Sequenase kit (U.S. Biochemicals).

Mutations that allowed conservation of efficient syncytium-forming activity of the envelope were subcloned by introducing the *Spe*I-*Bst*BI fragment (8285 to 8916) into the infectious molecular clone p34TF10 (31) (obtained from J. Elder through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health).

Cell transfection and syncytium formation assay. Transfection of feline CrFK/ID10/R fibroblasts (20) (gift of R. Osborne, Medical Research Council Retrovirus Research Laboratory, University of Glasgow), referred to in this article as CrFK cells, and syncytial assays were performed in a six-well plate, as previously described (22). Under these conditions, in untransfected cultures or in cultures transfected with plasmids containing nonfusogenic envelopes (such as the FIV PPR envelope), cells with more than five nuclei were rarely observed. For quantitative analysis of fusogenic ability of mutant envelopes, plasmids were purified by using the Promega Maxiprep system followed by phenol-chloroform extraction. Cells were transfected with 1 μg of DNA per well or incubated with transfection buffer without DNA (mock transfected), in triplicate. Cells were fixed and stained by the May-Grünwald-Giemsa method 48 h after transfection. Syncytia containing more than five nuclei were enumerated by microscopy with a 66-square grid.

Infectivity assays. CrFK cells were transfected in six-well plates with 3 μg of DNA of infectious molecular clone p34TF10 or mutant proviruses. All experiments were performed in duplicate. After 3 days the cells were transferred to 25-cm^2 flasks. The cells were split every 2 to 3 days and the reverse transcriptase (RT) activity of the culture supernatants was monitored by a microtest as described previously (11), with minor modifications. For quantification of results, 10 μl of RT reaction mixtures was distributed in duplicate on DE81 paper squares. After washing, ^{32}P activity was counted in a scintillation counter. For infectivity assays with cell-free virus, culture supernatants were collected 15 days after transfection, centrifuged, and filtered through 0.45-μm-pore-size filters. Supernatants were adjusted with medium to equivalent RT activity values, and 1 ml of supernatant was added, in duplicate, to 10^6 CrFK cells in suspension. Cells were incubated for 1.5 h at 37°C, washed with phosphate-buffered saline (PBS), and then plated in 25-cm^2 flasks and cultured in fresh medium. The cells were split 1:4 every 2 to 3 days, and RT activity in culture supernatants was measured.

To verify the sequences of the mutant viruses used for infections, RNA was prepared from supernatants by mixing 300 μl of each supernatant with 300 μl of urea lysis buffer (7 M urea, 0.35 M NaCl, 10 mM Tris HCl [pH 7.5], 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and performing phenol-chloroform extraction. After DNase digestion, RNA was reverse transcribed with random hexamers (Pharmacia) and Superscript Plus (Gibco BRL) by following the manufacturers' instructions. In other experiments, cell DNA was prepared for PCR by lysis of cells in 10 mM Tris (pH 8.3)–0.45% Nonidet P-40 (32). In every case, the *Spe*I-*Nde*I fragment of the FIV envelope gene was amplified by PCR and sequenced.

RIPA. Env processing was analyzed by pulse-chase followed by glycoprotein absorption on lentil lectin and radioimmunoprecipitation assay (RIPA), essentially as described previously (22). Briefly, 1.5×10^6 cells were plated on 10-cm-diameter petri dishes the day before transfection and were transfected with 15 μg of each plasmid. For two-point chase, 2.5×10^6 cells were transfected and split in two dishes the following day. Forty-two hours posttransfection, cells were harvested in methionine- and cysteine-deprived medium (ICN) for 1 h and then pulsed for 20 min with 100 μCi of Tran^{35}S-label (ICN) per ml. In some experiments, Pro-mix L-^{35}S labelling mix (Amersham) was used. After being washed with PBS, cells were chased for 0, 1, and 4 h in 4 ml of Dulbecco modified Eagle medium containing methionine and cysteine fivefold concentrated and supplemented with 10% calf serum. Cells were lysed in lectin-RIPA (L-RIPA) buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 1% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride), and supernatants were treated with 1:10 diluted volumes of 10× L-RIPA buffer. Cell lysates and treated supernatants were incubated overnight with 200 μl of Sepharose-lentil lectin (Pharmacia) suspended in L-RIPA buffer. After four washes with L-RIPA buffer, bound glycoproteins were eluted by incubation of beads with 400 μl of 0.5 M methyl-α-D-mannopyranoside (Sigma) in L-RIPA buffer for 2 h. Eluates were incubated overnight with a 1:100 dilution of pooled sera from FIV-infected cats. Immune complexes were precipitated with protein A (Boehringer), eluted by boiling in Laemmli buffer (14), and analyzed by SDS-polyacrylamide gel electrophoresis (5 to 15% gradient gel for cell-associated proteins and 10% gel for supernatants).

To analyze virion-associated proteins, 2 days after transfection of proviruses (15 μg of DNA/10-cm-diameter petri dish) cell cultures were labelled overnight with 100 μCi of Tran^{35}S-label (ICN) per ml. RIPA was performed on cell lysates or virus pellets. Supernatants were collected, centrifuged at $1,200 \times g$ for 10 min, and filtered through 0.22-μm-pore-size filters. Virions were pelleted by centrifugation for 3 h through a 25% sucrose cushion at 25,000 rpm (Beckman SW41 rotor). Pellets were dissolved in 300 μl of L-RIPA buffer. Samples were cleared by overnight incubation with serum from uninfected cats (diluted 1:100) and by precipitation with protein A. After centrifugation, supernatants were incubated overnight with a 1:100 dilution of FIV-infected-cat serum and a 1:200 dilution of cat serum directed to the C terminus of gp100. After precipitation with protein A, immunoprecipitates were analyzed by SDS–10% polyacrylamide gel electrophoresis. Gels were dried and exposed on a Molecular Dynamics Storage Fluor screen. Band radioactivity was measured with a PhosphorImager SI (Molecular Dynamics) and ImageQuant software.

ELISA. Nonapeptides corresponding to wild-type (peptide p237 [1]) or mutant PID loop sequences were synthesized by Neosystem Laboratories, Strasbourg, France. A peptide (catalog no. SP89029; Agence Nationale de Recherche sur le SIDA) corresponding to the sequence of the HIV-1 PID was used as a negative control. Peptide enzyme-linked immunosorbent assay (ELISA) was performed essentially as described previously (29). Peptides in 0.1 M carbonate buffer, pH 9.6, were applied to 96-well microplates (Dynatech Immunolon 2) (0.5 μg/well). After blocking wells with 3% bovine serum albumin, feline sera diluted in PBS–1% bovine serum albumin–0.1% Tween 20 (dilution buffer) were added and the mixtures were incubated for 2 h. After five washes with PBS–0.1% Tween 20, peroxidase-conjugated anti-cat immunoglobulin antibodies (Kirkegaard and Perry Laboratories, Inc.) were incubated for 1 h. The reaction product was visualized by using 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (0.4 mg/ml) and the optical density at 405 nm was read after 30 min. We first titrated all sera with each peptide to determine the optimal dilutions to be used in comparative and competition experiments. Sera from specific-pathogen-free cats were used in all the experiments to evaluate the background. Then we compared the reactivities of each serum with all the different peptides at a given dilution (1:100 for experimentally infected cats and 1:200 for naturally infected cats). For competition experiments plates were coated with 0.1 μg of peptide per well. Fifty microliters of diluted cat sera was mixed with increasing concentrations of the competitor peptide in 50 μl of dilution buffer and added in duplicate

TABLE 1. Amino acid sequences of the mutated PIDs

Envelope type and PID mutant	Sequence[a]
Nonfusogenic	
f3	CNQNQARC
f4	CNQNQRFC
f9	CNQNQTEC
f11	CNQNQSFC
f13	CNQNQVNC
fv	CNQNQVRC
n12	CRPWIFFC
n36	CGHVLFFC
n49	CLVGGFFC
n52	CRZFVFFC
n65	CPVGAFFC
n83	CRWDIFFC
n98	CZZSSFFC
Weakly fusogenic	
n10	CSTYTFFC
Strongly fusogenic	
f8	CNQNQWLC
fd	CNQNQFLC
fh	CNQNQLWC
fm	CNQNQPFC
n14	CEHQHFFC
n19	CSMGTFFC
n67	CLTDSFFC
n73	CELKNFFC
n92	CRPAAFFC

[a] Wild-type sequence: CNQNQFFC. Z, encoded by stop codon.

to wells. ELISA was continued as described above. Percent inhibition compared to that obtained with a control buffer sample was determined. Feline sera were provided by A. Moraillon (National Veterinary School, Maisons-Alfort, France). Sera from experimentally infected cats were obtained from cats infected with four isolates of FIV: Me, Wo, Le, and Be (16, 16a).

RESULTS

Fusogenic envelopes containing mutations in the PIDs. Fifteen clones of the envelope expression vector pTΔ20ds (see Materials and Methods) obtained by mutagenesis of the codons encoding the four N-terminal residues (pn) and 34 clones obtained by mutagenesis of the codons encoding the two C-terminal residues (pf) of the cysteine loop were used for transfection of CrFK cells and tested in a syncytium formation assay. Ten clones were able to induce syncytium formation. The sequences of the mutated domains of the fusogenic envelopes are shown in Table 1. Some other clones that failed to induce syncytium formation were also sequenced to analyze whether particular substitutions could account for the observed differences in envelope fusogenicity. Except for clones in which a stop codon was found, there were no apparent common features of clones with mutations maintaining and clones with mutations not maintaining envelope fusogenicity of the N-terminal portion of the PID (Table 1). Fusogenic envelopes presented extensive changes (affecting four residues) and a wide spectrum of mutations, including the introduction of acidic or basic amino acids (by clones pn14, pn73, and pn92). In contrast, mutations which introduced polar or charged amino acids in the two C-terminal positions of the cysteine loop caused the loss of envelope fusogenicity. These results suggest that the C-terminal part of the loop is subject to structural constraints, i.e., nonpolar amino acids are needed to conserve the viral functions of the PID. It is noteworthy that the PIDs of both FIV and HIV-1 contain hydrophobic residues in this position.

Quantitative evaluation of the number and size of syncytia induced after transfection of the mutant clones showed that nine mutant envelopes mediated syncytium formation with efficiency, although to a lesser extent than the wild-type (Table 2). Two mutant envelopes in particular, f8 and n67, induced syncytia similar in size to those induced by the wild-type envelope (Fig. 1).

Expression and processing of the fusogenic mutant envelopes. Expression and processing of the nine mutated envelopes were analyzed by transfection of CrFK cells with wild-type and mutant envelope expression vectors, followed by L-RIPA. Figure 2 shows the results of immunoprecipitations performed on cell lysates and supernatants collected immediately (at time zero [*T* 0]) after a 20-min pulse and 4 h later (*T* 4). For the supernatants, no signal was detected at *T* 0, and only the results obtained at *T* 4 are shown (Fig. 2). The 130-kDa Env precursors were expressed by all the mutants and were precipitated from cell lysates at *T* 0 (Fig. 2). The 4-h chase showed that the mutant Env precursors were cleaved into gp100 (SU) and gp36 (TM), like the wild-type envelope (Fig. 2). While a quantitative measurement of precursor cleavage and Env subunit production could not be obtained by L-RIPA, the results of several experiments taken together indicated that not all mutant envelopes were cleaved with the same efficiency. In particular, the n14 and n92 envelopes showed very efficient SU and TM production, while the processing of the fd, fh, and n73 envelopes appeared to be less efficient than that of the wild-type envelope. The SU was released into culture supernatants and could be detected at *T* 4 for all the mutants (Fig. 2).

Effect of PID mutations on infectivity. The nine *env* genes encoding the mutated PIDs were introduced into the FIV infectious molecular clone 34TF10 to generate mutant proviruses (pTn and pTf). After transfection of CrFK cells, RT activity was monitored in culture supernatants (Fig. 3). Most

TABLE 2. Syncytium formation induced by mutant envelopes

Envelope[a]	Sequence	No. of syncytia[b]	No. of nuclei (range)[c]
W	CNQNQFFC	4,435.2 ± 13.2	6–>100
f8	CNQNQWLC	2,714.8 ± 143.5	6–>100
fd	CNQNQFLC	532.4 ± 27.1	6–>100
fh	CNQNQLWC	814.0 ± 82.3	6–80
fm	CNQNQPFC	457.6 ± 65.0	6–80
n14	CEHQHFFC	1,298.0 ± 256.5	6–100
n19[d]	CSMGTFFC		6–60
n67	CLTDSFFC	3,964.4 ± 371.0	6–>100
n73	CELKNFFC	589.6 ± 189.9	6–60
n92	CRPAAFFC	1,694.0 ± 188.6	6–100

[a] CrFK cells plated in six-well plates were transfected in triplicate (in duplicate for the wild type [W]) with 1 μg of DNA of each clone per well.

[b] Syncytia were counted in five fields per well by using a grid (66 squares/well). Only syncytia with more than five nuclei were counted. Means ± standard deviations of the numbers of syncytia in three wells are shown. In the two wells with mock-transfected cells there was a total of five syncytia with more than five nuclei.

[c] See also Fig. 1.

[d] Syncytium formation induced by transfection of mutant clone pTn19 was quantitated in a separate set of experiments. The number of syncytia was approximately 40% of that observed with the wild-type envelope.

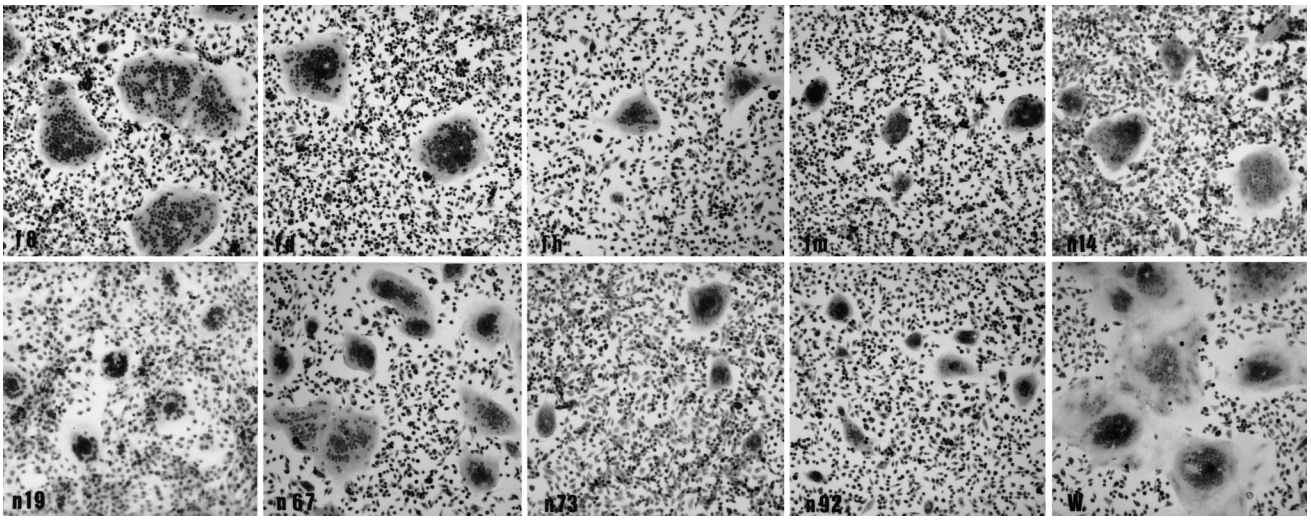

FIG. 1. Syncytium formation induced by mutant envelopes. CrFK cells in six-well plates were transfected with 1 μg of DNA of Env expression vectors. At 48 h posttransfection cells were fixed and stained by May-Grünwald-Giemsa coloration. Results of transfections with vectors encoding nine mutant envelopes (the names of the envelopes are indicated in the left corner at the bottom of each picture) and with the wild-type pTΔ20ds (W) are shown.

mutants showed a peak of activity early after transfection, and activity decreased progressively with cell passages (from three to five passages) and corresponded to transient protein expression from transfected genomes. In contrast, three clones were able to establish productive infection: pTn14 and pTn92 showed RT kinetics comparable to those of the wild-type clone, while pTn73 RT activity remained lower than that of the wild type (Fig. 3B). RNA and DNA were extracted from supernatants and cells after transfection with the three infectious mutant clones, and the *Spe*I-*Nde*I fragments of the envelope genes were amplified by RT-PCR (RNA) and PCR (DNA). Sequencing of the PCR products confirmed that the infectious viruses corresponded to the mutant clones.

Transfection of the nine mutant clones also resulted in extensive syncytium induction during the first 6 to 9 days (data not shown). This syncytial effect disappeared more slowly than the RT activity for the uninfectious clones, persisted for pTn73, and became more and more accentuated for pTn14 and pTn92. After 2 to 3 weeks, the infection became chronic and the cytopathic effect was attenuated, as observed with the wild-type virus.

The infectious clones were not the most efficient in mediating cell-to-cell fusion and syncytium formation: pTf8 and pTn67, which had the highest syncytium formation efficiency among the mutant viruses, were unable to give rise to a productive infection. For both mutant clones, RT activity, measured in supernatants 4 days posttransfection, was four times less than that for the wild-type clone (pTf8, $4.3 \times 10^5 \pm 0.4 \times 10^5$ cpm/ml; pTn67, $4.1 \times 10^5 \pm 0.8 \times 10^5$ cpm/ml; 34TF10, $16.7 \times 10^5 \pm 3.7 \times 10^5$ cpm/ml). Amplification by PCR of viral DNA from CrFK cells after incubation with pTf8 and pTn67 supernatants confirmed the inefficiency of cell-free mutant viruses in infecting cells (data not shown).

To further analyze this contrast in fusogenic properties and infectivity, we compared cell-associated and virion-associated proteins by RIPA after transfection of CrFK cells with the wild-type *env* gene, with mutant pTn14, which is infectious, and with pTf8 and pTn67, which are fusogenic but not infectious. Viral structural proteins were detected both in cell lysates (data not shown) and in virions (Fig. 4), indicating that complete viral particles were assembled and released in the super-

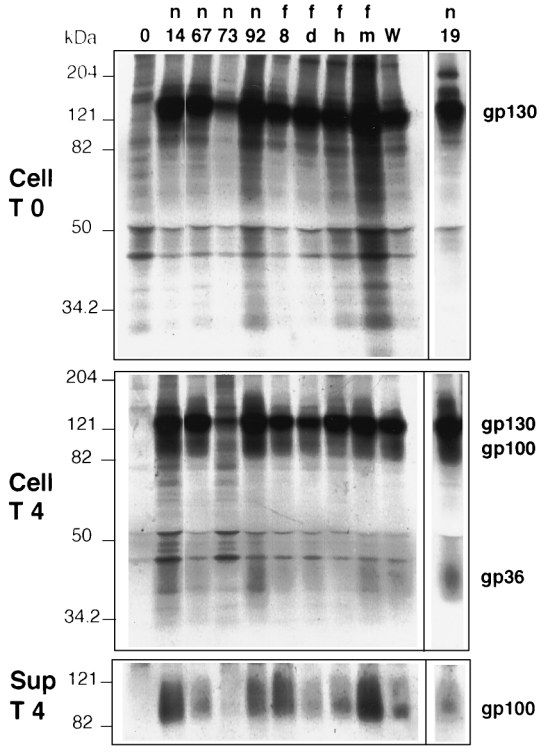

FIG. 2. Pulse-chase analysis of Env glycoproteins expressed upon transfection of CrFK cells with Env expression vectors containing the mutated or the wild-type (W) *env* genes. n19 envelope processing was analyzed in a different experiment and is shown on the right. The names of the envelopes are above the relevant lanes. Lane 0, mock transfection. L-RIPA was performed on cell lysates (Cell) or supernatants (Sup). The duration of chase before harvest of cell-free culture media is indicated on the left. Precipitated Env glycoproteins are indicated on the right; molecular mass standards are indicated on the left. The Env precursor gp130 was precipitated immediately after the pulse (*T* 0) from cell lysates for the wild-type and the mutants; after 4 h the processing of the Env precursor was indicated by the detection of the SU gp100 in the lysates. The TM gp36 appeared as a diffuse band, more evident for clones pn14 and pn92 in this experiment. gp100 was precipitated from supernatants after a 4-h chase (bottom). The expression of the n73 envelope was low, and the gp100 that was precipitated from the supernatant is barely visible.

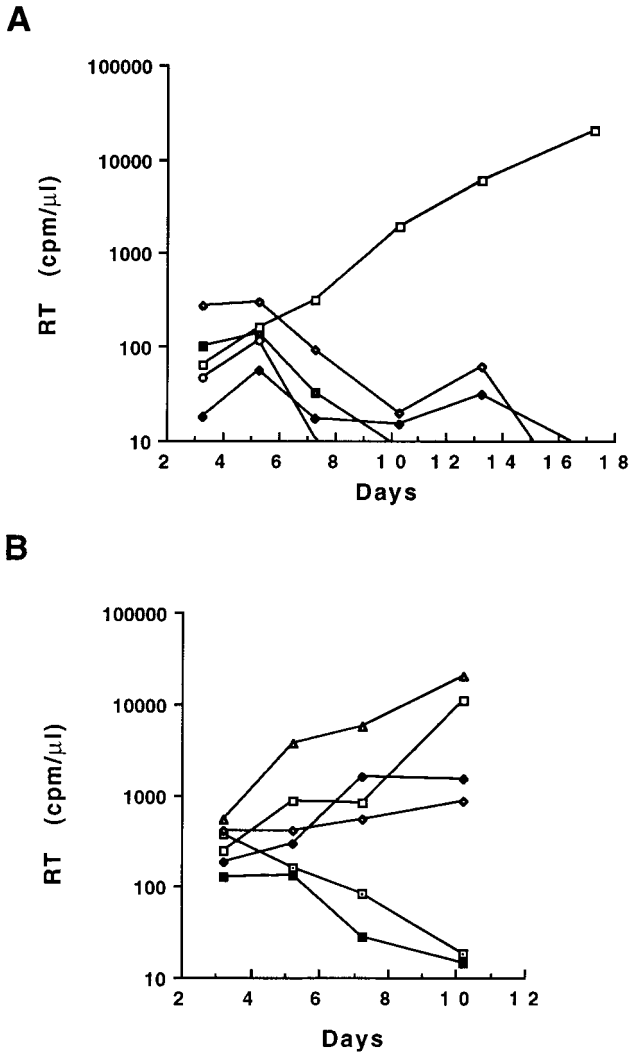

FIG. 3. Kinetics of RT activity in the supernatants of cells transfected with FIV molecular clones containing mutations in the sequence encoding the PID or with the wild-type 34TF10 (□). Three to four experiments were performed for each mutant, with similar results. (A) Transfection of molecular clones containing mutations in the codons encoding the two C-terminal amino acids of the PID loop. RT activity declined after 5 days posttransfection for all the mutants. ○, pTf8; ◆, pTfd; ■, pTfh; ◇, pTfm. (B) Transfection of molecular clones containing mutations in the codons encoding the four N-terminal amino acids of the PID loop. Transfection of three clones, pTn14 (△), pTn73 (◇), and pTn92 (◆), resulted in the establishment of a productive infection, as indicated by the increase in RT activity released in the supernatants. ■, pTn19; ⊡, pTn67. RT values are the means of two independent measurements, corrected by substraction of the backgrounds of the mock transfections (49 ± 5.3 cpm/μl [A] and 66 ± 13.9 cpm/μl [B]).

natants for all clones. Immunoprecipitated proteins were quantified with a phosphorimager. The gp100 signal was normalized for each virus in relation to the p64 and p52 bands corresponding to RT proteins. The ratios of the levels of normalized gp100 in mutants to those in wild-type viruses were 1.16, 1.08, and 0.98 for pTf8, pTn67, and pTn14, respectively. Therefore, defects in virus assembly and envelope incorporation which could underly differences in infectivity were not detected in these assays.

The abilities of free pTn14 and pTn92 viruses to infect CrFK cells were analyzed by using cell culture supernatants collected 15 days after transfection. Equal amounts of RT activity from wild-type and mutant supernatants were used for infections.

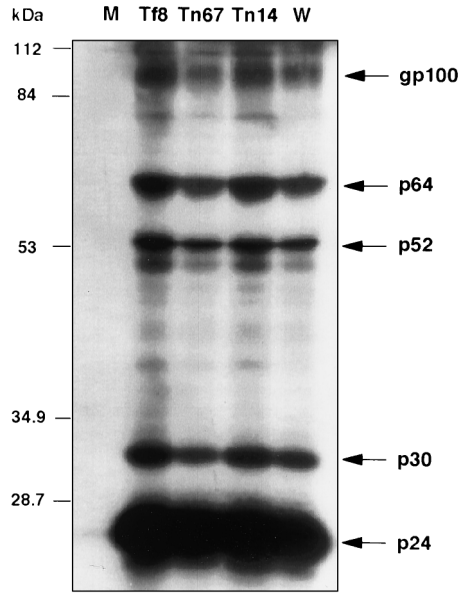

FIG. 4. RIPA analysis of viral proteins from virions pelleted from cell-free supernatants after cell transfection with three mutant molecular clones or with 34TF10 (W). M, Pellets from mock-transfection supernatants. Viral proteins are indicated by arrows: p24 corresponds to the Gag CA protein; p30, p52, and p64 correspond to the Pol proteins IN and RT; and gp100 corresponds to the Env SU. Molecular mass standards are indicated on the left.

Measurement of RT activity in cell supernatants after infection showed that both mutant viruses established infection (Fig. 5). The kinetics of infection of pTn92 were quite similar to those of the wild type, while pTn14 RT activity was detected with a delay of approximately 4 days and rose later to the level of wild-type activity. Syncytium formation developed parallel to RT activity, although the cytopathic effect was much more pronounced in cells infected by 34TF10 than in cells infected by pTn92 or pTn14.

The observation that dissemination of the pTn14 virus in cell cultures was similar to or even more rapid than that of the wild-type virus after transfection (Fig. 3B), but slower after free-virus infection (Fig. 5), suggests that cell-to-cell transmis-

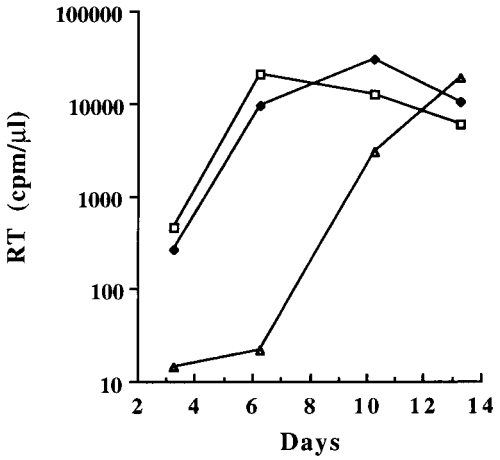

FIG. 5. Kinetics of infection of CrFK cells by mutant viruses pTn14 (△) and pTn92 (◆), in comparison to the wild-type 34TF10 (□). Cells were infected with supernatants containing equal amounts of RT activity (see Materials and Methods). RT values are the means of two independent measurements after subtraction of the background of the mock infection. These results were reproducible in different experiments.

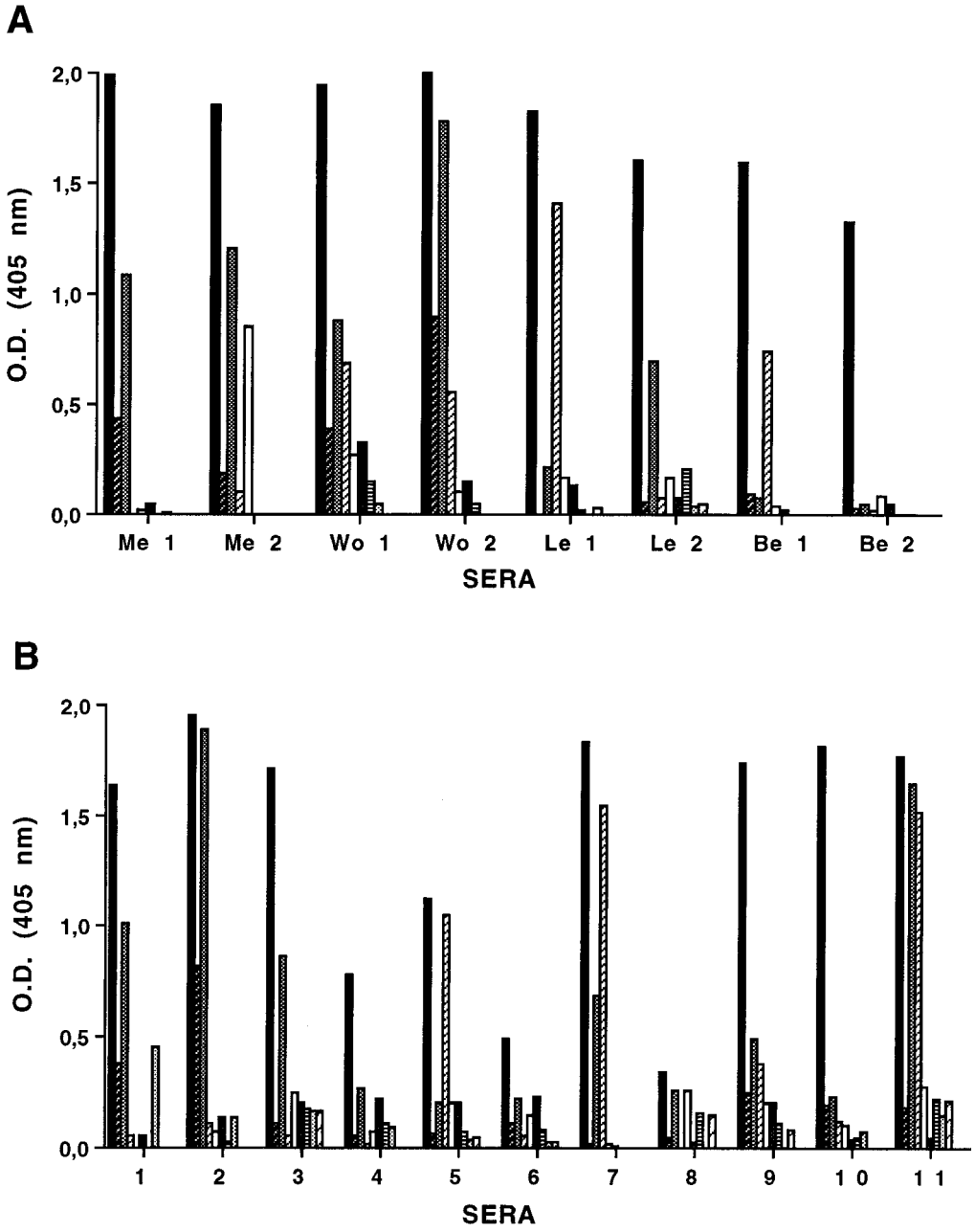

FIG. 6. ELISA performed on peptides representing the wild-type PID or mutant sequences with sera from 8 experimentally infected (diluted 1:100) (A) and 11 naturally infected (diluted 1:200) (B) cats. Optical densities (O.D.) were obtained for FIV peptides after subtracting the background (reactivity against an unrelated peptide). Cat serum numbers and infecting isolates are indicated below the abscissas. ■, wild type; ▨, f8; ▩, fd; ▨, fh; □, fm; ▨, n14; ▤, n19; ▨, n67; ▨, n73.

sion could be the preferential mode of transmission for pTn14. Alternatively, the sustained high level of virus production observed after transfection with pTn14 could reflect the fact that this mutant was much less cytopathic than the wild-type virus during the first days following transfection.

Effects of mutations on the immunological properties of the PID. In order to analyze the effects of mutations on the immunological properties of the PID, we measured the reactivities of synthetic nonapeptides corresponding to the wild-type sequence or to the mutated domains of the nine fusogenic envelopes with sera from FIV-infected cats. Sera from 8 cats experimentally infected with four different strains of FIV (Fig. 6A) and from 11 naturally infected field cats (Fig. 6B) were tested by a peptide ELISA. The peptide corresponding to the wild-type sequence was recognized by all sera and was strongly reactive with all sera from the experimentally infected cats and most sera from the naturally infected cats, confirming a previous report from our laboratory (1). In comparison to this peptide, all the peptides containing mutations showed reduced reactivity; in particular, the peptides with mutations in the N-terminal portion of the loop displayed little or no reactivity with both sets of sera from infected cats (Fig. 6). The peptide corresponding to the mutated domain of the n92 envelope could not be analyzed by peptide ELISA due to the high background found with uninfected-cat sera. However, competition assays showed that there was no cross-reactivity between the wild-type and n92 peptides (Fig. 7). Figure 7 shows the results of an inhibition experiment performed with serum, Wo

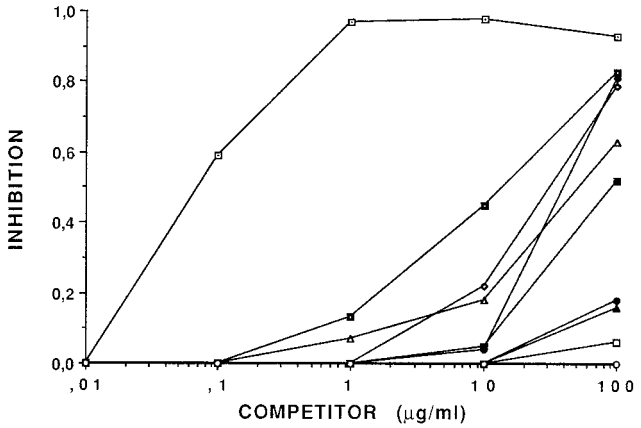

FIG. 7. Inhibition of antibody binding to the wild-type peptide (⊡) by mutant peptides. Wild-type and mutant peptides were used as competitors for binding of cat serum with the wild-type peptide applied to microplates at 0.1 μg/well, as described in Materials and Methods. The concentration of competitor peptides is expressed in micrograms per milliliter. ⊡, wild type; ◆, f8; ◘, fd; ◇, fh; ■, fm; □, n14; ▲, n19; △, n73; ●, n92; ○, HIV-1 PID.

2, from a cat experimentally infected with FIV Wo, by using the wild-type peptide adsorbed to plates. In this experiment, of the N terminus mutants, only n73 peptide partially inhibited antibody binding to the wild-type peptide at concentrations 100 times higher than those of the wild-type peptide. Of the C terminus mutants, the fd peptide, with one amino acid change, displayed a significant (>40%) degree of competition at a concentration 10 times higher than that of the wild type. Other competition assays were performed with sera from experimentally or naturally infected cats, and in some assays mutant peptides were bound to microplates and wild-type peptide was used as the competitor (data not shown). These experiments confirmed that most mutated peptides did not cross-react with the wild-type peptide. The finding that peptides containing C-terminal mutations, such as fd or fh, reacted with some sera from infected cats was not surprising, the amino acid changes being conservative and restricted to one or two residues. Nevertheless, it is interesting that, in the case of HIV-1, it has been reported that the two C-terminal hydrophobic residues of the PID loop do not have a determining role in PID immunoreactivity with HIV-1-positive sera (18).

DISCUSSION

The PID of FIV is the most conserved antibody binding region of the envelope. The sequence contained between the two cysteines that characterize this domain does not show variation among viral isolates in spite of a strong humoral response that persists during the course of infection. In this work we showed that the FIV envelope tolerates extensive mutations in this sequence giving rise to infectious mutant viruses. Given the potential for variation of lentivirus populations, structural constraints exerted on viral protein therefore seem insufficient to account for the conservation of the PID sequence observed in vivo.

Another interesting observation made during this study was that viral infectivity did not correlate with fusogenic ability of the mutant envelopes. For example, mutants pTf8 and pTn67, whose envelopes were properly processed and induced a stronger syncytial effect after transfection than the infectious mutants pTn92 and pTn14, were not able to establish a productive infection. Similar dissociation of envelope fusogenic activity and viral infectivity has also been described for HIV-1. For example, deletions in the cytoplasmic domain of the HIV-1 TM, which resulted in an inefficient incorporation of the truncated envelopes into virions, caused a loss of the ability of mutant envelopes to mediate virus entry into cells but did not block envelope-mediated cell fusion (6). However, we did not observe obvious differences in virion-associated envelopes between noninfectious and infectious viruses by RIPA. In another study, HIV-1 envelopes containing point mutations in the C-terminal half of the TM ectodomain efficiently mediated entry into lymphocytes but were defective in inducing syncytia (3). The mechanism involved in the loss of mutant virion infectivity observed in the present study is unclear. Different mechanisms can be hypothesized, such as diminished efficiency of virion-cell fusion or postfusion events, including reverse transcription (33) due to a modified envelope conformation, or deficient incorporation of envelope complex in virus particles caused by changes in envelope processing. Neither mechanism was detected by the methods used in this study.

This work addressed both the virological function and the immune reactivity of the PID. We demonstrated that different variants of this domain permit the expression of functional envelopes. However, substitutions of the two C-terminal phenylalanines of the PID which retained the fusogenic ability of the envelope were restricted to nonpolar amino acids. Moreover, no mutation in these residues that maintained viral infectivity was found: the change of a single amino acid was sufficient to cause a loss of infectivity (clones pTfd and pTfm). It is noteworthy that computer modeling performed on chimeric PIDs, in which the FIV sequence between the cysteines was replaced with the corresponding sequence of visna virus or caprine arthritis encephalitis virus (CAEV), showed differences in steric bulk for the C-terminal position, while the HIV-1–FIV chimeric domain showed a good match with the FIV domain in this position (21). Indeed, of these three chimeras, only the HIV-1–FIV chimera retained fusogenic activity. These data together with present experimental results suggest that the two hydrophobic C-terminal amino acids contribute to determining adequate folding of the loop and/or are important for the interaction of the PID with SU or other parts of the envelope.

In contrast to the C-terminal portion of the PID loop, which appeared to be "locked," changes in the N-terminal amino acid sequence of the PID loop were functionally tolerated but modified the reactivity of this domain with sera from FIV-infected cats. Little or no cross-reactivity between peptides containing the mutations and the peptide corresponding to the wild-type sequence was found. These results suggest that the virological function of the PID is independent of its immunological properties. The conservation of both the spatial structure and the primary sequence of the PID could be the result of independent selective processes. Functional constraints could explain the conservation of the three-dimensional structure: previous reports have shown that the cysteines and the loop structure defined by them are essential for precursor processing and the maturation of the envelope glycoproteins of FIV and HIV-1 (5, 21, 30). Nevertheless, different sequences could assume similar spatial configurations, providing an explanation for why certain modifications of the sequence contained between the two cysteines can permit the conservation of envelope function. The results reported here support the hypothesis that functional constraints alone could not account for the conservation of the amino acid sequence.

Although it is not possible at this stage of the experimental work to formally exclude the possibility that a minimal advantage in replication conferred by the canonic sequence of the

PID would select and fix this sequence, it is improbable that the natural genetic variability of lentiviruses does not produce diverse sequences capable of ensuring equivalent virological functions of the PID. The conservation of the immunodominance of the PID in all the lentiviruses, including HIV-1, may reflect a selective pressure exerted by antibodies directed to this region, which favor the virus life cycle. Indeed, our work shows that mutations introduced in the PID affect antibody recognition more frequently than envelope function. In the case of HIV-1, anti-PID human monoclonal antibodies have been shown to enhance virus infection of a human T-cell line by a complement-dependent mechanism (24, 25) and infection of human lymphocytes by an antibody Fc-mediated mechanism (7). In the case of infection of goats by CAEV, we have shown that the reactivity of sera from infected goats with the CAEV PID was associated with the presence of clinical arthritis (2). In vivo relevance of antibody-dependent enhancement of viral infection is suggested by several studies (reviewed in reference 15), and mechanisms of antibody-mediated dissemination in host lymphoid organs were proposed in a recent report, which showed that HIV virions coated with antibodies and attached to follicular dendritic cells were still infectious (12). Should anti-PID antibodies favor viral infection and dissemination, the immunodominance of the enhancing structure may be expected to be conserved and its primary sequence preferentially fixed in the virus population. The potential impact of this hypothesis is to be considered in the design and development of envelope-based vaccine trials for lentiviral infections.

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