Structural characterization of the feline-immunodeficiency-virus envelope glycoprotein 36 ectodomain for the development of new antivirals

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In the fight against the human HIV, new targets are being explored, such as the proteins involved in the process of fusion of the virus with the host cell. Recently, the first generation of fusion inhibitors (enfuvirtide), targeting gp41 (virus envelope glycoprotein 41), has become commercially available. However, this promising class of drugs has to be improved in respect of their efficacy and bioavailability. Considering the strong homologies between HIV and FIV (feline immunodeficiency virus), as well as the highly conserved structure of the transmembrane envelope protein among species, FIV represents a relevant model of pre-screening studies for HIV. Taking into account (i) sequence homologies between the ectodomain of HIV gp41 and FIV gp36 (envelope glycoprotein 36), (ii) structural data available for gp41 and (iii) the fact that synthetic peptides derived from gp36 are effective inhibitors

of FIV infection, we designed several peptides derived from gp36 sequence. We checked that these peptides had the same structural features as the corresponding peptides from gp41 HIV by CD, analytical ultracentrifugation and ¹H–²H (hydrogen–deuterium) exchange combined with MS. By combining this latter technique with surface-plasmon-resonance assays, we identified the amino acid residues of the C-terminal region of the ectodomain of gp36 that are critical for interaction with the N-terminal region. This gave clues for therapy and vaccines against FIV, thus providing helpful data for treatments against HIV.

Key words: feline immunodeficiency virus (FIV), glycoprotein 36 (gp36), fusion inhibitor, hydrogen–deuterium (¹H–²H) exchange, mass spectrometry (MS).

INTRODUCTION

Until now, neither curative nor preventive treatment against human immunodeficiency virus (HIV) is available, even though combined anti-(viral protease) and anti-(viral reverse transcriptase) treatment can dramatically decrease the viral load. Owing primarily to either toxicity of these molecules and/or virus mutation leading to resistance, interest in alternative therapeutic approaches is growing. New targets are being explored [1], such as the proteins involved in the process of fusion of the virus with the host cell.

The HIV fusion process is well known and involves gp120 (envelope glycoprotein 120) and gp 41 [2]. Cellular receptor and co-receptor recognition by gp120 induces conformational changes in gp41. The N- and C-terminal regions of the gp41 ectodomain form a six-helix bundle that makes up the core of a trimer-of-hairpins. It brings the viral and cellular membranes into the close proximity necessary for fusion, resulting in the subsequent delivery of the viral genome into the host cell [3]. Fusion inhibitors block virus in a prefusion state and prevent membrane fusion. In 1992, for the first time, a synthetic peptide containing amino acid residues derived from gp41 was reported to block virus-mediated cell–cell fusion processes [4]. Since then, numerous studies were undertaken to understand better the fusion process and to design peptides with better inhibition efficiencies [5]. Recently, the first generation of this new class of drugs, the fusion inhibitor enfuvirtide [6], has become commercially available. This large peptide (36 amino acid residues in size), also called T-20 or Fuzeon[®], exhibits both low toxicity and high efficacy. Although it significantly decreases the viral load, it lacks oral availability,

needs to be injected in large amounts and is rather expensive. Clearly the efficacy and bioavailability of this promising class of drugs has to be improved.

The interaction between the N- and the C-terminal regions of the gp41 ectodomain was described in HIV-1, where both regions show a 4,3 hydrophobic (heptad) repeat, a sequence motif characteristic of coiled coils. Biophysical studies first identified this interaction with the synthetic peptides N51 (residues 540–590) and C43 (residues 624–666) [7]. These peptides associate to form a stable α -helical discrete trimer of heterodimers. This association was confirmed by crystallography studies on peptides N36 (residues 546–581) and C34 (residues 628–661), showing a hydrophobic cavity (residues 565–577) formed by highly conserved amino acids in different HIV-1 strains, and hydrophobic amino acids from C34 interacting with this cavity (residues 628–635) [3]. The structure was also solved by crystallography after expressing two segments: residues 541–590 fused with a transcription-factor-GCN4 isoleucine zipper fragment and residues 624–665 [8].

Other lentiviruses, such as FIV (feline immunodeficiency virus) and simian immunodeficiency virus, share remarkable homology with HIV. FIV is a natural infection of domestic cats that results in an acquired immunodeficiency syndrome resembling HIV infection. The worldwide prevalence of FIV infection in domestic cats has been reported to range from 1 to 28%. Hence, an effective FIV vaccine or therapy will have an important impact on veterinary medicine [9].

In view of the strong homologies between HIV and FIV, as well as the highly conserved structure of the transmembrane envelope protein among species, FIV would seem to represent a relevant

Abbreviations used: AUC, analytical ultracentrifugation; ESI-MS, electrospray-ionization MS; FIV, feline immunodeficiency virus; gp36, FIV envelope glycoprotein 36; ¹H–²H exchange, hydrogen–deuterium exchange; MS-MS, tandem MS; SPR, surface plasmon resonance; TFA, trifluoroacetic acid.

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model of pre-screening studies for HIV. Furthermore, studies using cats would allow faster development of fusion inhibitors, because these clinical trials are easier to perform. Use of FIV as a model for HIV would also help to improve *in vivo* efficiency and/or stability, which are crucial issues in human therapy.

Taking into account (i) sequence homologies between the ectodomain of HIV gp41 and FIV gp36, (ii) structural data available for gp41 and (iii) the fact that synthetic peptides derived from gp36 are effective inhibitors of FIV infection [10–12], we designed several peptides derived from the gp36 sequence. We checked that these peptides had the same structural features as the corresponding peptides from gp41 HIV by CD, analytical ultracentrifugation and ^1H – ^2H (hydrogen–deuterium) exchange combined with MS. By combining this latter technique with SPR (surface plasmon resonance) assays, we identified the amino acid residues of the C-terminal region of the ectodomain of gp36 that are critical for the interaction with the N-terminal region. This provides clues for therapy and vaccines against FIV, thereby providing helpful data for treatment of HIV.

EXPERIMENTAL

Materials

Peptide N70 (28 amino acid residues from the the transcription factor GCN4 plus 42 amino acid residues derived from gp36 sequence 650–691) was chemically synthesized using the Fmoc (fluoren-9-ylmethoxycarbonyl) technology (Dictagene, Lausanne, Switzerland). Peptides W35Q, W35K, W18D, W15T, I15Q, W15I, K15E and K15Q corresponding to sequences 723–757, 733–767, 733–750, 723–737, 728–742, 733–747, 738–752 and 743–757 respectively, derived from the gp36 C-terminal region, were chemically synthesized using the same manufacturing process (Pepscan Systems, Lelystad, The Netherlands; NeoSystem, Strasbourg, France). TFA (trifluoroacetic acid), sodium phosphate, citric acid, $^2\text{H}_2\text{O}$ and pepsin were obtained from Sigma–Aldrich. Acetonitrile was from SDS, ZI de Valdonne, Peypin, France.

Digestion with pepsin

Peptide digestions were performed in an ice bath at 0°C . A pepsin solution ($1\ \mu\text{g}/\mu\text{l}$) was prepared in $0.1\ \text{M}\ \text{H}_3\text{PO}_4$, pH 1.6, and precooled at 0°C in the ice bath. The peptides were digested for 30 s at pH 2.5 in $70\ \text{mM}$ phosphate buffer with an enzyme/peptide ratio of 1:1 (w/w).

Sequence assignments

The proteolytic fragments of non- ^2H -labelled peptides were separated on a reverse-phase C_{18} Interchrom column ($1\ \text{mm} \times 100\ \text{mm}$) with a linear gradient of acetonitrile [5–70% (v/v) acetonitrile/water (9:1, v/v)/0.03% TFA (solvent B)] for 30 min at a flow rate of $50\ \mu\text{l}/\text{min}$. Prior to injection the column was equilibrated with 95% solvent A (water containing 0.03% TFA) and 5% solvent B for 20 min. Peptide assignments were performed by direct MS–MS (tandem MS) analysis.

Preparation of C-terminal peptide–N70 complexes

Stock solutions were prepared in 0.1% TFA and then diluted in $70\ \text{mM}$ phosphate buffer, final pH 7.6, for N70 and each C-terminal peptide, except for W35K, which was diluted in $40\ \text{mM}$ citrate buffer, final pH 4.5. The complexes (1:1, mol/mol) were formed and incubated in an ice bath at 0°C just prior to analysis.

^1H – ^2H exchange of peptides

W35Q or W35K (244 pmol) with or without N70 were diluted 20-fold in $70\ \text{mM}$ ^2H -labelled phosphate buffer (p ^2H 7.6) or in $40\ \text{mM}$ citrate buffer (p ^2H 4.5) and incubated for various periods of time at 0°C . Isotopic back-exchange was quenched by addition of a precooled $2\ \text{M}$ HCl solution in order to decrease the p ^2H of the mixture to 2.5. Samples were loaded on a Protein MacroTrapTM cartridge (Michrom Bioresources, Auburn, CA, U.S.A.) and desalted with water supplemented with 0.1% TFA. To limit isotopic back-exchange during the analysis, the column was precooled and maintained at 0°C in an ice bath. Peptides were eluted with 70% of acetonitrile/water (9:1, v/v)/0.1% TFA and analysed by ESI–MS (electrospray-ionization MS).

^1H – ^2H exchange of peptide fragments

W35Q or W35K (244 pmol) peptide in the presence or absence of N70 was diluted 20-fold in ^2H -labelled buffer. ^2H -labelling was performed at 0°C for 2–30 s in order to exchange all the accessible amide ^1H atoms of the peptides with ^2H . Isotopic back-exchange was quenched by the addition of $6\ \mu\text{l}$ of pepsin solution, pH 1.6. After pepsin digestion, ^2H -labelled peptide fragments were rapidly concentrated and desalted on a peptide MacroTrapTM column at a flow rate of $300\ \mu\text{l}/\text{min}$. ^2H -labelled peptides were separated under the conditions used for sequence assignments. To limit isotopic back-exchange, the column was precooled in an ice bath and maintained at 0°C . ^2H -labelled peptides were analysed by ESI–MS.

ESI–MS and ESI–MS–MS analysis

Kinetics of ^1H – ^2H exchange were performed on an API III+ triple quadrupole mass spectrometer equipped with an ionspray source (PerkinElmer Sciex, Thornhill, Ont., Canada). The electrospray probe tip was held at 5 kV and the declustering potential was set at 80 V. Data were processed using Masspec 3.3 software and the reconstructed molecular-mass profiles were obtained with a deconvolution algorithm from the m/z mass spectrum containing multiple charge states. Sequence assignment and ^1H – ^2H exchange experiments on peptic fragments were performed on a quadrupole ion trap mass spectrometer Esquire 3000+ (Bruker Daltonik, Bremen, Germany) equipped with an ion-spray source. MS and MS–MS experiments were carried out with a capillary voltage set at 4 kV and an end-plate offset voltage at 500 V. The nebulizer gas (N_2) pressure was set at 69 kPa (10 lbf/in 2) and the dry gas flow (N_2) at 8 litres/min at a temperature of 250°C . Mass spectra were analysed by using Bruker Daltonics DataAnalysis 3.0 and Biotools 2.1 software.

CD analysis

CD spectra of peptides at a final concentration of $12\ \mu\text{M}$ were collected at room temperature in a JASCO spectrophotometer over a 195–260 nm wavelength range and in 1 nm steps. Three spectra were averaged for each peptide. The helical content was estimated using the CD spectra deconvolution software.

Analytical ultracentrifugation

The analytical-ultracentrifugation experiments were done with a Beckman XL-1 centrifuge equipped with an An-60Ti rotor at 50 000 rev./min and at 20°C . The sample concentration was $12\ \mu\text{M}$.

SPR binding assays using BIAcore technology

The SPR measurements were performed in a BIAcore 3000 instrument (Biacore AB, Paris, France). N70 was covalently coupled

to a CM5 sensor chip (research grade) via its primary amine groups (amine coupling kit, Biacore AB). The carboxymethylated dextran surface was activated by injection of a mixture of 0.2 M *N*-ethyl-*N'*-(diethylaminopropyl)carbodi-imide and 0.05 M *N*-hydroxysuccinimide. The peptide was then injected in 10 mM sodium phosphate, pH 7.6, containing 240 mM NaCl. Activation time, peptide concentration (0.1–0.5 mg/ml) and contact time were adjusted to the desired extent of immobilization. An immobilization level ranging between 150 and 1500 RU (resonance units) was achieved: the lowest level was used for kinetic analysis and the highest for binding assays. Residual *N*-hydroxysuccinimide esters were blocked by the injection of 1 M methanolamine hydrochloride, pH 8.5. All immobilization steps were performed at a flow rate of 5 μ l/min.

The control flow cell was prepared by performing the coupling reaction in the presence of coupling buffer alone. It was used to assess non-specific binding of injected C-terminal peptides to the surface of the sensor chip, as well as refractive-index changes resulting from changes in bulk properties of the solution [13–15]. Control sensorgrams were automatically subtracted from sensorgrams obtained with immobilized N70 in order to yield true binding responses [13–15].

Binding assays were performed at 25 °C with 70 mM sodium phosphate, pH 7.4, containing 240 mM NaCl and 0.005 % P20 surfactant (Biacore AB) as running buffer. C-terminal peptides were injected at several concentrations and different flow rates over immobilized N70. The surface was regenerated with a 1 min pulse of 3 M guanidinium chloride. W35Q, W35Q* (in which alanine replaced Phe⁷⁴⁴, Tyr⁷⁴⁵, Ile⁷⁴⁷ and Ile⁷⁴⁸ in the W35Q sequence) and W18D were dissolved in 40 mM sodium phosphate, pH 7.6, containing 240 mM NaCl and 0.005 % P20 surfactant. W35K was dissolved in 40 mM citrate, pH 4.5, containing 240 mM NaCl and 0.005 % P20 surfactant. Kinetic analysis was performed by injecting several concentrations of C-terminal peptides over immobilized N70. The kinetic parameters k_a and k_d (association and dissociation rate constants) were determined simultaneously using a global data analysis program (BIAeval 3.1 software; Biacore AB), which constrains the kinetic rate constants to a single value for each set of curves. R_{max} , the maximal capacity of the surface, was floated during the fitting procedure. The closeness of the fit is described by the statistical value χ^2 , which represents the mean square of the signal noise and should be lower than 10.

RESULTS

Design of the peptides enabling modelling of the interaction site in FIV

To select peptides derived from the N- and C-terminal regions of the ectodomain of FIV gp36, the HIV-1 gp41 (BH10 strain) and FIV gp36 (WO strain) sequences were aligned using ClustalW multiple alignment [16] (Figure 1). The N-terminal peptide was designed by focusing on the HIV-1 region 551–592, including the hydrophobic pocket described by Chan et al. [3] (residues 565–577), which should correspond to 650–691 sequence in FIV according to sequence alignment. Furthermore, using the GeneMine software, a helical hydrophobic pocket was predicted in the middle of this region (670–677). A peptide derived from FIV sequence 650–691 was synthesized, adding the first 28 residues of a trimeric GCN4 isoleucine zipper fragment to the N-terminus to promote the formation of a helical structure. This chimera was similar to that used to obtain the X-ray structure of the ectodomain from HIV-1 gp41 [8]. Three amino acid residues of the GCN4 moiety were changed to increase the solubility, as proposed by Eckert et al. [17]. This peptide was called N70.

Figure 1 Sequence alignment of HIV-1 gp41 and FIV gp36

The sequences of the ectodomains of HIV-1 gp41 (BH10 isolate) and of FIV gp36 (WO strain) were aligned. The (known or predicted) hydrophobic pockets are in bold. The sequences at the origin of peptides W35Q, W35K and W18D are underlined. The region selected for the N70 is also underlined.

Figure 2 CD spectra of W35Q and N70

The CD spectra were obtained for separated peptides, W35Q (—) and N70 (.....), at a concentration of 12 μ M and for the peptides mixed in equimolar amount at a final concentration of 12 μ M (---).

We also synthesized two peptides derived from the C-terminal region of the ectodomain of FIV gp36. These peptides were expected to interact with the N-terminal region, W35Q (723–757) and W35K (733–767). Shorter peptides were also synthesized in order to further investigate the N–C interactions.

Biophysical characterization of the peptides and of their interactions

As expected, the N-terminal peptide alone, N70, adopts a helical structure. Its CD spectrum (Figure 2 and Table 1) displayed two minima at 208 nm and 222 nm, which are characteristic of α -helices. The helical content was estimated to be $94 \pm 6\%$, with a molar ellipticity of -21.9×10^3 degrees $\cdot$ cm² $\cdot$ dmol⁻¹ at 222 nm using CD spectra deconvolution software. Moreover, AUC (analytical ultracentrifugation) showed the presence of one species with an experimental sedimentation coefficient of 1.7 ± 0.1 S (at a sedimentation velocity of 42 000 rev./min). This value is very close to the theoretical coefficient of a trimeric protein

Table 1 CD molar ellipticity of N70 alone or in the presence of C-terminal peptides

The molar ellipticity at 222 nm of N70 alone at a concentration of 12 μ M or in solution with C-terminal peptides in equimolar amounts was obtained from CD spectra collected at room temperature over a 195–230 nm wavelength range.

Peptidic solution	Molar ellipticity at 222 nm (10^3 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$)
N70	–21.9
+ W35Q	–31.8
+ W35K	–31.5
+ W18D	–25.6
+ W35Q*	–22.6

under these analytical conditions (1.9 S). This suggests that N70 assembles into a trimer.

CD spectra of the C-terminal peptides (W35Q and W35K) were characteristic of a random structure without any element of secondary structure (Figure 2 for W35Q). AUC gave values of 0.66 S and 0.42 S for W35Q and W35K respectively, close to the calculated coefficients (0.61 S and 0.49 S) for monomeric proteins in their respective buffers.

When each C-terminal peptide was individually mixed in equimolar amounts with N70, dramatic changes could be observed in CD spectra and AUC curves when they were compared with those of N70 alone. The CD spectrum of the N70/W35Q mixture (Figure 2) showed an increase in molar ellipticity at 208 nm and 222 nm compared with N70 alone, from -21.9×10^3 up to -31.8×10^3 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ at 222 nm, indicating an increase in the α -helical content. The mixture of N70 and W35K showed a similar increase of ellipticity, up to -31.5×10^3 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ at 222 nm (results not shown).

The AUC data for the mixture showed the disappearance of the species at 1.7 S, corresponding to the trimeric N70. In contrast, a peak with a sedimentation coefficient of 2.5 S should correspond to a hexamer (calculated coefficient 2.6 S), suggesting the formation of a complex comprising three N70 and three W35Q peptides.

The global interaction between the C-terminal peptides and N70 was further characterized using ^1H – ^2H exchange experiments combined with MS. These experiments took into account the fact that the amide hydrogen atoms in a protein can exchange with ^2H in a ^2H -labelled buffer at rates that depend on their accessibility and on their participation in secondary structures. Association of ^1H – ^2H exchange with MS has been widely used in structural biology for more than 10 years, providing information on protein structure, protein dynamics [18,19] and protein–protein interactions [20].

After incubation of W35Q in a ^2H -labelled buffer for 2–30 s, the peptide mass increased from 4352 Da to 4380 Da (Figure 3). This mass increase corresponded to the exchange of 82 % of the 34 amide hydrogen atoms. This high rate of fast exchange was consistent with the CD data showing no defined structure of the peptide alone in solution, thus giving an easy access to the solvent for most of its amide hydrogen atoms. The remaining amide hydrogen atoms are likely due to back-exchange during the MS analysis. When the incubation in the ^2H -labelled buffer was done in the presence of N70, the mass of W35Q was 4365 Da, indicating that only 38 % of its amide hydrogen atoms had undergone exchange. A protection of W35Q by N70 from the solvent was thus clearly shown, confirming an interaction between both peptides and the appearance of structure in W35Q. Similarly, a protection of N70 by W35Q was also observed: 28 % of N70

Figure 3 Mass spectra of W35Q

The ESI mass spectra of W35Q were obtained before deuteration of the peptide (—), after 30 s ^2H -labelling of the peptide alone (---) and after 30 s ^2H -labelling of the peptide mixed in equimolar amount with N70 (.....). Molecular weight $\equiv$ molecular mass.

amide hydrogen atoms underwent exchange after 30 s incubation in a ^2H -labelled buffer, whereas only 22 % were exchanged in the presence of W35Q. The lower exchange rate of N70 (28 %) compared with W35Q (82 %) when each peptide is alone can be explained by the trimeric helical structure of N70.

We observed similar protection against ^2H exchange when N70 and W35K were incubated together in a ^2H -labelled buffer (results not shown).

All of these experiments demonstrated that the peptides from FIV gp36 designed from HIV/FIV sequences behave the same way as the N- and C-terminal peptides from HIV-1 gp41. The N-terminal peptide adopted a trimeric helical structure, and three C-terminal peptides interact to form a trimeric complex of heterodimers. Therefore, the FIV peptides can be used to characterize further the structural aspects of the interaction between N- and C-terminal peptides that is required for the fusion process.

Fine characterization of the interaction with N70

To determine which residues of the C-terminal peptides are involved in the interaction with N70, ^1H – ^2H exchange, proteolysis in conditions of slow back-exchange (low pH and low temperature) and MS were combined. W35Q and W35K peptides were digested with pepsin, which is active at pH 2.5 and 0°C. Since this enzyme is not highly specific, each proteolytic fragment of both peptides was identified by MS-MS. A complete coverage of both sequences was obtained. After incubation in a ^2H -labelled buffer for 0–30 s of W35Q, alone or in the presence of N70, W35Q was digested with pepsin. The evolution of ^2H incorporation was measured for each fragment in both conditions.

When W35Q was ^2H -labelled alone, fragments 1–8, 1–10 and 28–35 quickly incorporated ^2H , reaching an equilibrium after only 2 s deuteration, in agreement with the absence of secondary structure observed for the C-terminal peptides. Furthermore the exchange rate for these fragments was not influenced when W35Q was incubated in the presence of N70, indicating that there was no interaction between these fragments and N70 (Figure 4).

When W35Q was ^2H -labelled alone, fragments 23–27 and 23–28 also showed a fast ^2H incorporation. But, in contrast with the previous fragments, ^2H incorporation was inhibited in the presence of N70 (see the kinetics in Figure 4 and the mass spectra in Figure 5A). The absence of ^2H labelling indicated a strong interaction between this region of W35Q and N70, together with the appearance of secondary structure of W35Q.

Fragment 9–22 showed an intermediate situation, with partial protection by N70 (see the kinetics in Figure 4 and mass spectra in

Figure 4 ^2H -labelling kinetics of fragments from W35Q in interaction with N70

W35Q was incubated in a ^2H -labelled buffer for 0–30 s. Back exchange was quenched at low pH and low temperature and the peptide was digested with pepsin for 30 s. After online separation using HPLC, the mass of the fragments was measured using ESI-MS. ^2H ('Deuterium') incorporation was obtained for each fragment: 1–8 or 28–35 (—), 9–22 (---), 23–27 or 23–28 (.....).

Figure 5 Mass spectra of peptic fragments of W35Q

The mass spectra of fragment 23–27 (**A**) and fragment 9–22 (**B**) were obtained under the conditions described in the legend to Figure 4. W35Q was non- ^2H -labelled (—), 30 s ^2H -labelled alone (---) and in the presence of N70 (.....). Abbreviation: Intens, intensity.

Figure 5B), thus indicating a weaker interaction of both peptides in this region and/or a lower content of secondary structure in W35Q.

These results are summarized in Figure 6, where the sequence of this peptide is presented. Three regions have different behaviours towards exchange protection by N70: 1–10 and 29–35 showed a high exchange, 11–22 a mild exchange and 23–28 a low exchange.

The same experiments were performed with W35K, giving the same cleavage sites in the central region with pepsin. Fragment 1–12, equivalent to region 11–22 in W35Q, also showed a moderate exchange when N70 was present with W35K. Fragments 13–17 and 13–18, equivalent to 23–27 and 23–28 in W35Q, were also weakly exchanged. As was found for fragment 29–35 in W35Q,

Figure 6 Protection level of different regions of W35Q and W35K

Sequences of W35Q and W35K showing the regions with low protection (white background), mean protection (light-grey background) and high protection (dark-grey background) against ^1H – ^2H exchange by N70.

Figure 7 Model of the FIV gp 36 ectodomain

This model was based on the X-ray structure of HIV-1 gp41 [8], using the programs ONO [31] and PyMOL [32]. The N70 peptide is shown in blue. W18D is shown in two regions: 733–744 in red and 745–750 in yellow.

fragment 19–35 was not protected. The sequence of W35K, presented in Figure 6, shows the similar exchange behaviour for identical regions in both peptides.

Identification of an essential region for the interaction

The study of the interaction of W35Q and W35K with N70 indicated that a common region in both C-terminal peptides presented a mild or low exchange in the presence of N70. To characterize this region further, a shorter peptide, called W18D, was synthesized, corresponding to the region 11–29 in W35Q (745–750 in the gp36 sequence; see Figure 1). This peptide, together with N70, is shown in Figure 7 on a model of FIV gp36 structure based on the X-ray structure of HIV-1 gp41 [8].

Whereas CD analysis of W18D alone showed no secondary structure, the molar ellipticity of W18D in the presence of N70 increased to -25.6×10^3 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ at 222 nm (Table 1). This increase was smaller than with W35Q and W35K. A global protection against ^1H – ^2H exchange when N70 and W18D were mixed: 11 amide hydrogen atoms out of 17 were protected after 30 s of ^2H -labelling.

After digestion with pepsin, four fragments covering the W18D sequence were identified using tandem MS. Two of them, 13–18 (YEIIMD sequence, shown in yellow in Figure 7) and 13–17, were strongly protected when N70 was in the presence of W18D. In contrast, fragment 1–12 showed only a moderate exchange, like the equivalent fragments found in W35Q and W35K.

Five overlapping peptides covering the sequence of W35Q: W15T, I15Q, W15I, K15E and K15Q, corresponding to sequences 723–737, 728–742, 733–747, 738–752 and 743–757 respectively, were also synthesized. None of them showed any protection against exchange when in the presence of N70.

Identification of amino acids essential for the interaction between the N-terminal and the C-terminal regions

The sequences of several strains of FIV and HIV-1 viruses were aligned at the Swiss Institute of Bioinformatics using the BLAST network service, showing that three amino acid residues in the 745–750 region are strongly homologous. This is also the case for the phenylalanine residue located upstream. To probe the role of these amino acids we replaced Phe⁷⁴⁴, Tyr⁷⁴⁵, Ile⁷⁴⁷ and Ile⁷⁴⁸ with Ala in W35Q sequence and called the corresponding synthetic peptide 'W35Q*'. In contrast with the other C-terminal peptides, the mixture of W35Q* with N70 did not induce any increase in molar ellipticity measured at 222 nm by CD (Table 1). Furthermore, no protection by N70 against ¹H–²H exchange was observed for W35Q* (results not shown).

These results showed the absence of interaction between N70 and W35Q* and confirmed the essential role of these mutated amino acid residues.

Binding of the C-terminal peptides to immobilized N70

The binding of the C-terminal peptides to immobilized N70 was studied by SPR using BIAcore technology. Immobilized N70 retained its ability to bind to the three peptides, namely W35Q, W35K and W18D (Figure 8A). The length of the shortest peptide, W18D, was sufficient to promote binding to N70, although it bound to N70 to a lower extent than W35Q and W35K. The association rate of the three peptides with N70 was similar, but the dissociation rate of the three complexes was different. W35K and W18D exhibited a faster dissociation rate than W35Q (Figure 8A). The plateau was higher for W35K than for W35Q, although their masses are equivalent, because they are dissolved in different buffers (W35K in citrate buffer and W35Q in phosphate buffer), which have a different refractive index.

Binding of short or mutated C-terminal peptides to immobilized N70

Mutation of Phe⁷⁴⁴, Tyr⁷⁴⁵, Ile⁷⁴⁷ and Ile⁷⁴⁸ to alanine within W35Q inhibited its binding to immobilized N70 by 97% (Figure 8B). Short C-terminal peptides (15 residues) K15E and K15D, which contain the YEIIMD sequence, did not bind to immobilized N70 (results not shown). This suggests that the sequence YEIIMD was necessary, but not sufficient, for binding to immobilized N70.

Kinetic analysis of interactions of C-terminal peptides with immobilized N70 by SPR

The optimal flow rate for kinetic analysis was assessed by injecting the three peptides at a single concentration, but at different flow rates (20, 40, 60 and 80 μ l/min) over immobilized N70. No significant variation in the association phase was observed between 40 and 80 μ l/min suggesting that mass transfer was not a limitation for the evaluation of kinetic parameters in this

Figure 8 Sensorgrams for the binding of the C-terminal peptides to immobilized N70

Sensorgrams obtained by injection of 500 nM W35Q (.....) in phosphate buffer, 500 nM W35K (—) and 1 μ M W18D (---) over immobilized N70 at a flow rate of 20 μ l/min for 5 min (A). Injection of W35Q (.....) and W35Q* (— · —) over immobilized N70 at a flow rate of 20 μ l/min for 5 min (B). Abbreviation: RU, resonance units.

flow-rate range. A flow rate of 60 μ l/min was selected for kinetic experiments.

To discriminate between different models of interaction, C-terminal peptides were injected at a single concentration over immobilized N70 for 3, 4 or 5 min. Dissociation rates varied as a function of contact time, indicating that linked reactions took place during the binding. Indeed when the reactions are linked, the apparent dissociation rate decreases as more stable complexes are formed during a longer injection phase [21]. The mechanism of interaction of the C-terminal peptides with immobilized N70 could thus be accounted for either by a bivalent-analyte model or by a two-state-reaction model. A contact time of 4 min was chosen for kinetic analysis, and spontaneous dissociation of complexes in buffer flow was monitored for 5 min.

Kinetic data were collected by injecting different concentrations of the three C-terminal peptides (2.5–500 nM for W35Q and W35K, 50–1000 nM for W18D) over immobilized N70 (results not shown). According to kinetic analysis with BIAeval 3.1 software, experimental sensorgrams were best fitted to the two-state reaction model. This model accounts for conformational change in the complex, a more and more stable complex being formed over time. It corresponds to the following equations:

Conformational changes in ligand or complex do not normally give a response in the BIAcore technology, because they are not associated with a change in mass. This is the reason why the association rate constant describing the second state of the reaction was expressed in s^{-1} and not in $M^{-1} \cdot s^{-1}$. The two sets of rate constants and the apparent affinity constant, K , calculated by the BIAeval software, were reported in Table 2.

Table 2 SPR data of the interaction between immobilized N70 and C-terminal peptides

The binding of the C-terminal peptides to the immobilized N70 was studied by SPR. Kinetic and affinity constants were evaluated by fitting binding data to the two state reaction model in the BIAeval 3.1 software.

Peptide	k_{a1} ($M^{-1} \cdot s^{-1}$)	k_{d1} (s^{-1})	k_{a2} (s^{-1})	k_{d2} (s^{-1})	$1/K [= 1/(k_{a1}/k_{d1}) \times (k_{a2}/k_{d2})]$ (M)	χ^2
W35Q (723–757)	2.33×10^{-5}	4.21×10^{-2}	4.08×10^{-3}	2.92×10^{-3}	1.29×10^{-7}	0.672
W35K (733–767)	0.82×10^{-5}	5.21×10^{-2}	4.72×10^{-3}	3.14×10^{-3}	4.22×10^{-7}	0.754
W18D (733–750)	0.0056×10^{-5}	2.02×10^{-2}	2.6×10^{-3}	1.41×10^{-3}	1.95×10^{-5}	0.0629

W35Q and W35K bound to N70 with similar kinetic and affinity constants, except for the association rate governing the formation of the first complex, which was decreased by a factor of 3 for W35K. W35Q bound initially to N70 with higher affinity (1.81×10^{-7} M) than W35K (6.35×10^{-7} M). When the affinity of the whole binding process, including the formation of the two complexes, was evaluated by the apparent affinity constant $1/K$ (M) defined as $1/(k_{a1}/k_{d1}) \times (k_{a2}/k_{d2})$, it appeared that W35Q bound to N peptide with higher affinity than W35K.

The difference is much more pronounced for W18D, which interacted initially with N70 at a much lower rate than the two other C-terminal peptides (Table 2). The value of the association rate constant for the formation of the first complex between W18D and the N70 was ≈ 150 - and ≈ 420 -fold lower than that for W35K and W35Q respectively. W18D also bound initially to the N peptide with a much lower affinity (360×10^{-7} M) than W35Q and W35K. The value of the apparent affinity constant, K , taking into account the two steps of the interaction, differed by a factor of 150 when compared with W35Q and 46 when compared with W35K. This confirmed that W18D bound to the N70 with a lower affinity than W35Q and W35K, although the two complexes formed by this peptide were more stable.

DISCUSSION

Peptides derived from the C-terminal region of the ectodomain of HIV-1 gp41 have been used to inhibit the virus-cell fusion [3,17,22,23] or for immunization [24]. Synthetic or recombinant peptides derived from the ectodomain have also been used to solve the HIV-1 gp41 structure, which is a six-helix bundle consisting of a trimeric coiled-coil core (N-terminal region) with three exterior helices (C-terminal region) packed in the grooves [3,8,25].

To explore new therapeutic approaches against FIV and to demonstrate that this virus could actually be a model useful in the fight against HIV, we designed peptides derived from FIV gp36 ectodomain according to sequence alignments of FIV gp36 and HIV-1 gp41 and to the three-dimensional structure of gp41.

A chimera formed by a GCN4 moiety followed by 32 amino acid residues derived from the N-terminal region of gp36 was synthesized. This region was chosen for its homology with the N-terminal region of gp41 from which peptides had been derived for structural and functional studies. Furthermore, the gp36 region was predicted to possess a hydrophobic pocket similar to that of gp41. This deep hydrophobic cavity revealed by the crystal structure [3], and which possessed highly conserved amino acid residues, was reported to be a target for drug development [17].

Using the biophysical techniques CD, AUC and 1H - 2H exchange combined with MS, this N70 peptide derived from gp36 was shown to adopt a trimeric helical structure. This is consistent with the formation of a fully helical soluble trimeric peptide (IQN17), which had been obtained by fusing the same GCN4 fragment with an N-terminal portion of gp41 encompassing the hydrophobic pocket [17].

On the basis of sequence alignment and gp41 X-ray crystal structure, C-terminal peptides (W35Q and W35K) from gp36 believed to interact with N70 were designed. When alone in solution, each of these peptides lacked secondary structure, which is consistent with the random-coil conformation already observed for peptides from the C-terminal region of gp41 ectodomain [26]. When each C-terminal peptide was mixed with N70, stable, trimeric complexes of heterodimers were formed, similar to those formed by the peptides from gp41. The identical structural behaviour of FIV gp36 and HIV-1 gp41 validate the choice of the FIV gp36 sequence to study further the mechanism of the fusion process.

A minimal region (W18D from residues 733–750) belonging to the C-terminal part of gp36 was identified as playing a major role in the interaction with the N-terminal part. Within this region, the 745–750 residues were shown to be crucial for the interaction, as they were highly protected by N70 against 1H - 2H exchange.

The SPR experiments confirmed that the 18 amino acid residues of W18D were sufficient to bind to N70, although they did not promote a high affinity binding. The mechanism that best described the interaction involves a conformational change in the first complex formed, leading to a more stable complex. The first complex between W18D and N70 formed at a lower rate than the complexes established with W35Q and W35K. Furthermore, the rate of formation of the second complex was also decreased for W18D compared with W35Q and W35K. This suggests that additional sequences present in W35Q and W35K play a role first by increasing the association rate with N70 and then by increasing the rate at which conformational changes leading to the formation of a more stable complex occur. In contrast, when the complex is formed, these additional sequences do not seem to be critical for its stabilization because they were not protected by N70 against exchange.

After alignment of the gp41 and gp36 sequences (Figure 1), it appears that the homologous gp41 region of W18D (631–648) shares part of its sequence with T20 (638–673), the most critical region of W18D (745–750) being homologous with a region (643–648) fully belonging to T20. From the gp41 X-ray crystal structure it appears that the amino acid residues derived from T20 do not interact with the hydrophobic pocket in the N-terminal region. By contrast, the homologous gp41 region (631–642) of fragment possessing the first 12 amino acid residues of W18D (733–744), which was shown to play a less critical role in the complex stabilization, interacts with the hydrophobic pocket. It thus appears that W18D probably interacts with N70 through the hydrophobic pocket and through a more critical region whose homologue is also targeted by T20.

We unsuccessfully attempted to decrease the size of the C-terminal peptides by designing five peptides of 15 amino acid residues covering the W35Q sequence. Although one of them (K15E) possesses the critical region, it did not interact with N70. The other peptides possessing only a portion of W18D did not interact either. These results obtained with shorter peptides

confirm that the critical region (745–750) and a region interacting with the hydrophobic pocket are not only sufficient, but also necessary, for a peptide to interact with N70. Medinas et al. [11] analysed the *in vitro* activity of several 35-amino-acid peptides derived from the C-terminal region of gp36 by their ability to prevent syncytium formation. One of them, with a sequence very similar to that of W35K (because this was a different strain, Leu⁷⁶² was changed into isoleucine), presented one of the lowest EC₅₀ values (20 nM). Two others having the same size, but shifted by one and two amino acid residues upstream (Asp⁷³² and Gly⁷³¹), had the lowest EC₅₀ (12 nM) values among the peptides they studied.

The next step in the present study will be to test the *in vitro* activity of W18D. If it shows an activity similar to that of the peptides discussed above, the next step would be to investigate peptidomimetic strategies. Indeed, the difficulties met with T20 are mainly due to its peptidic nature. Its bioavailability is limited, on account of extensive proteolysis, which requires the injection of large amounts of the peptide. Moreover, its large size (36 amino acid residues) makes it also rather expensive to produce. This is the reason why it would be interesting to design peptidomimetics. These metabolically stable peptide analogues mimic the peptide producing the same types of interactions with the target without proteolysis. Especially since the X-ray crystal structure of HIV-1 protease was solved, peptidomimetics have been designed to inhibit this target and are currently approved for the treatment of HIV infection [27]. With this objective, Eckert et al. [17] used IQN17 and mirror-image phage display, which let them identify cyclic, D-peptide inhibitors of HIV-1 infection. A retroinverso analogue of an octapeptide derived from the C-terminal region of gp36 was also shown to display an antiviral activity against FIV infection [28].

The design of a peptidomimetic analogue of W18D should be done after modelling the three-dimensional structure of the complex it forms with N70 by using the gp41 structure as a model. This peptidomimetic could be tested for its *in vitro* and *in vivo* activity. Within the framework of these tests, the feline model is interesting, because clinical studies are easier to handle in cats. If this therapeutic strategy based on peptidomimetics development decreases FIV load, it could become a major pre-screening step in the research of new therapies against HIV. The results obtained in the present study might also be of potential interest for vaccinal strategies. One of the current approaches to attenuate viruses used for vaccination is to introduce mutations within genes responsible for virulence [29]. Our results showed that the mutation of four amino acid residues, Phe⁷⁴⁴, Tyr⁷⁴⁵, Ile⁷⁴⁷ and Ile⁷⁴⁸ to alanine in the sequence of W35Q completely destabilized its interaction with N70. It is reasonable to postulate that the mutation of some of them would dramatically decrease virus infectivity, as it has already been shown for other parts of this transmembrane envelope gp36 [29,30]. In such a vaccinal context, the cat appears again to be an interesting model in researching a vaccine against human AIDS. In addition to the evidence of the protection induced by the attenuated virus, trials on cats would enable to establish the very sensitive risk/benefit balance, especially for these RNA viruses with a high mutation rate.

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