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Dissection of seroreactivity against the tryptophan-rich motif of the feline immunodeficiency virus transmembrane glycoprotein

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Abstract

Immunogenicity of the tryptophan-rich motif (TrpM) in the membrane-proximal ectodomain of the transmembrane (TM) glycoprotein of feline immunodeficiency virus (FIV) was investigated. Peptide 59, a peptide containing the TrpM of the TM of FIV, was covalently coupled to Qβ phage virus-like particles (Qβ-59) in the attempt to induce potent anti-TrpM B cell responses in cats. All Qβ-59 immunized cats, but not cats that received a mixture of uncoupled Qβ and peptide 59, developed antibodies that reacted with a same epitope in extensive binding and binding competition assays. The epitope recognized was composed of three amino acids, two of which are adjacent. However, Qβ-59-immune sera failed to recognize whole FIV in all binding and neutralization assays performed. Furthermore, no reactivity against the TrpM was detected by screening sera from FIV-infected cats that had reacted with TM peptides, confirming that this epitope does not seem to be serologically functional in the FIV virion. The data suggest that TrpM may not be a suitable target for antiviral vaccine design.

Keywords: FIV; Env; TM glycoprotein; VLP; Trp-rich motif; Peptide; Neutralizing antibodies; Immunogenicity

Introduction

The transmembrane (TM) glycoproteins of many fusogenic viruses possess a stretch unusually rich in tryptophans (Trp) and other aromatic amino acids (Trp-rich motif; TrpM) at their pretransmembrane domains, leading to postulate that such a stretch plays a key part in the important functions these molecules exert in virus–cell surface interactions (reviewed in Peisajovich and Shai, 2003). Indeed, for the TM glycoproteins most intensively investigated under this respect, human immunodeficiency type-1 (HIV-1) and vesicular stomatitis viruses, mutagenesis studies have demonstrated that an intact TrpM is essential for effective virus entry into cells (Jeetendra et al., 2003; Muñoz-Barroso et al., 1999; Robison and Whitt, 2000; Salzwedel et al., 1999). Moreover, in HIV-1, certain synthetic peptides that inhibit entry and neutralizing monoclonal antibodies 2F5 and 4E10 interact with the TM glycoprotein at sites that partially or nearly entirely overlap the TrpM (Chan and Kim, 1998; Zwick et al., 2001), indicating that this site can be an interesting target for antiviral strategies.

A similarly located TrpM is also present in the TM glycoprotein of feline immunodeficiency virus (FIV), and two lines of evidence have recently shown that it plays a critical role in virus entry. First, short synthetic peptides containing the region were found to be powerfully inhibitory for FIV replication and to be dependent for their activity on conservation of the Trp residues (Giannecchini et al., 2003). Second, mutants carrying severe disruptions of the TrpM adsorbed normally onto feline lymphoid cells but

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failed to enter, although mutants with minor changes in the motif entered cells with reduced efficiency (Giannecchini et al., 2004). It was therefore of interest to investigate whether the TrpM might represent a suitable target for immunological anti-FIV strategies.

In the present report, immunization of cats with an appropriate FIV peptide conjugated to virus-like particles (VLP) of the Qβ phage, used to enhance B-cell reactivity against the peptide (Lechner et al., 2002), led to the generation of antibodies that specifically bound a conformation-dependent epitope within the TrpM peptide in enzyme-linked immunosorbent assays (ELISA). However, the TrpM was found not to be serologically functional in the context of the FIV virion, as shown by failure of the immune sera thus raised to recognize the virus in binding and neutralization assays and by failure of FIV-infected cat sera to react with the motif in binding and binding competition assays. These findings suggest that the TrpM of FIV cannot be easily exploitable in devising antibody-based antiviral strategies.

Results

Antibodies to selected TM-derived peptides in peptide 59-immunized cats

We wanted to produce antibodies against the TrpM of FIV (Fig. 1). However, peptide 59, used to this purpose in previous studies, had failed to induce a humoral response in mice (Massi et al., 1997). In order to enhance the immunogenicity of peptide 59, we coupled it to VLP derived from the bacteriophage Qβ (Qβ-59; Fig. 2). It has previously been shown that antigens coupled to these VLP are highly immunogenic (Fehr et al., 1998; Jegerlehner et al., 2002a; Lechner et al., 2002; Pumpens et al., 1995). Specific-pathogen-free (SPF) cats were subsequently immunized with Qβ-59 (78 μg of Qβ-59 per cat carrying 2.5 μg of peptide 59). To determine whether such an immunization had generated an antipeptide 59 antibody response, preimmune and postimmune sera were diluted 1:100 and tested in ELISA plates coated with peptide 59, as well as with peptides 58 and

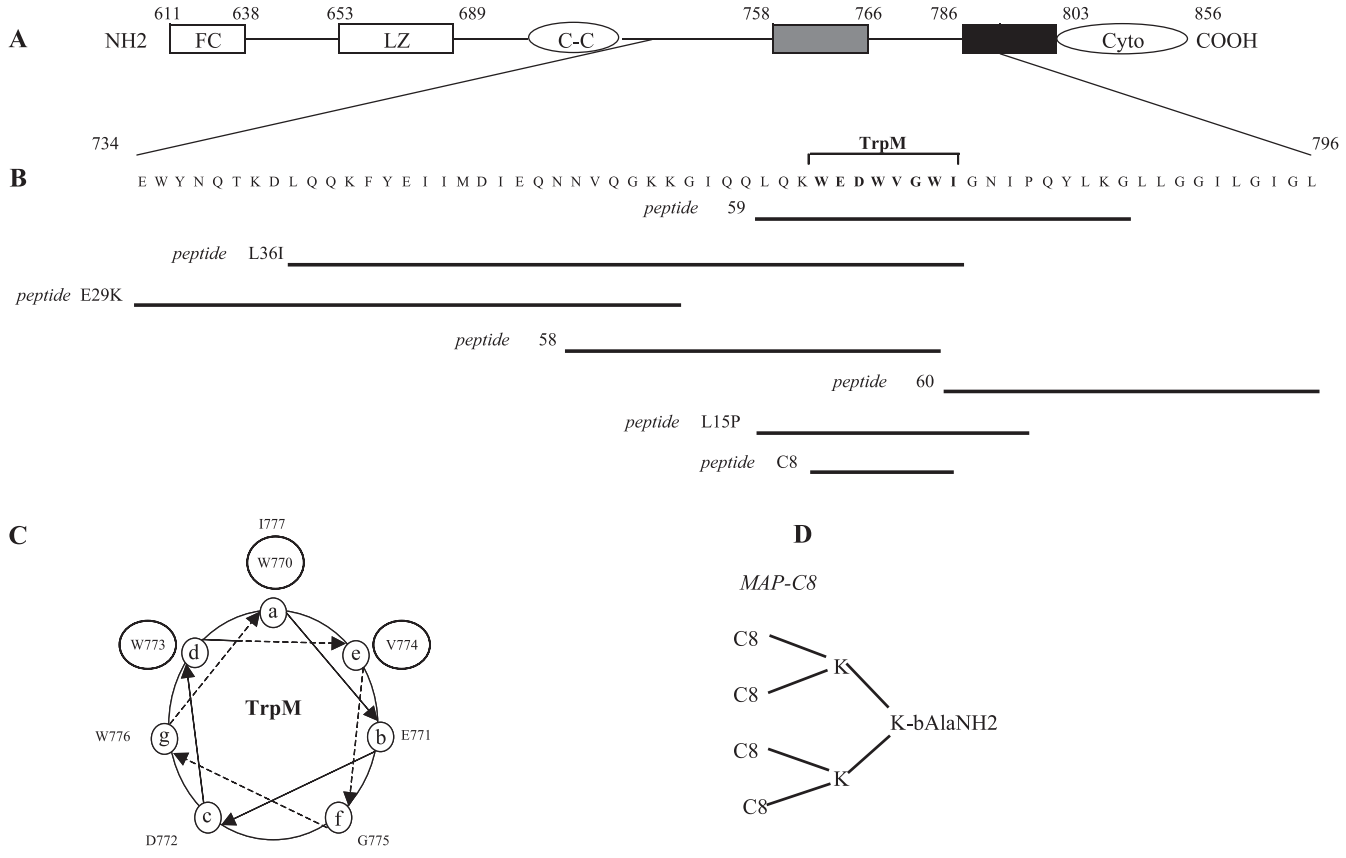

Fig. 1. Peptides used. (A) Schematic representation of the TM glycoprotein of FIV. Superscript figures denote amino acid positions starting with the first methionine of Env, according to the sequence of clone 34TF10 of FIV Petaluma (Talbott et al., 1989). TM domains are indicated as previously described (Richardson et al., 1998): FC, fusion peptide; LZ, leucine zipper region; C–C, cysteine loop; gray box, membrane-proximal α-helix; solid box, membrane-spanning segment; Cyto, cytoplasmic region. (B) Amino acid sequence of the TM in the tract used for modeling the synthetic peptides investigated. The amino acids forming the TrpM are in bold. (C) Helical wheel projection of the TrpM; the three amino acids essential for functionality of the B epitope recognized by immunizing cats with Qβ-59 are circled. (D) Schematic representation of the MAP-C8 used in serological assays for detection of anti-TrpM antibodies.

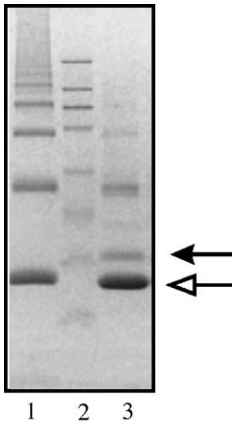

Fig. 2. SDS-PAGE analysis of the efficiency of coupling peptide 59 to Qβ. Twenty micrograms of Qβ (lane 1) or Qβ-59 were analyzed on a 12% SDS-PAGE gel under reducing conditions, and the relative intensity of coupled Qβ (solid arrow) and uncoupled (empty arrow), corresponding to a molecular mass of 14 kDa, was compared after Coomassie Blue staining and densitometric analysis. Lane 2: Molecular mass markers.

60, that overlapped the N- and C-terminal half of peptide 59, respectively (Fig. 1). No evidence of antibody reactivity could be detected in any of the test samples with any of the three peptides (Table 1). We then ran similar assays using as an antigen peptide L36I, that is, an adjacent and partially overlapping 36-mer that contained a sequence of 11 amino acids identical to the N-terminal portion of peptide 59 at its C-terminal (Fig. 1). Use of this larger peptide led to the demonstration of substantial antibody reactivity in all the animals immunized with Qβ-59 but in none of the preimmune sera or of the sera obtained from the animals injected with a mixture of uncoupled peptide and VLP (Table 1). As shown by Fig. 3, the antibodies detected by peptide L36I became first evident at the time of the third inoculum and reached the highest levels after the fourth dose. At this time, they ranged in titer between 1:200 and 1:400 in individual animals but subsequently declined rapidly and remained low in spite of boosting (data not shown). Epitope density on VLP surface has been shown to play a significant role in the induction of a strong IgG response in mice, leading to hypothesize that the higher the epitope density, the stronger the stimulus for B cells (Jegerlehner et al., 2002b). It is likely, therefore, that improving coupling efficiency between Qβ and peptide 59 might lead to higher and more durable antipeptide antibody titers. Unfortunately, the physicochemical features of the peptide chosen, especially its low solubility in water, did not allow to improve coupling efficiency, at least with the protocol used in the present study.

When similar ELISA tests were performed with the 29-mer E29K, no reactivity was detected in any of the test sera. Because this peptide encompassed most of the sequence of peptide L36I but lacked the tract this shared with peptide 59 (Fig. 1), this clearly suggested that, as expected, the L36I-reactive antibodies in the Qβ-59-immunized cats were specific for the TrpM-containing segment.

Antibodies to the TrpM in peptide 59-immunized cats

In these experiments, we looked for a confirmation that the antipeptide L36I antibodies detected in Qβ-59 immunized cats were indeed directed against peptide 59 and, at the same time, investigated whether they were specific for the TrpM contained in the latter. This was done by examining whether short peptides that contained the TrpM alone (peptide C8) or the TrpM flanked by few amino acids on one side (peptide 58) or both sides (peptide L15P) could directly bind antibody activity and compete with anti-L36I peptide serum reactivity present in all Qβ-59-immunized cats. As controls, we used two peptides of comparable length, one that reproduced a tract of the TM glycoprotein just outside the TrpM (peptide 60; Fig. 1) and the other a scrambled version of the TrpM (peptide $C8_{scr}$). As summarized in Table 1, all the direct binding assays conducted with these peptides were consistently negative. On the other hand, the competition experiments were extremely informative. Indeed, as illustrated by Fig. 4, which depicts the results of a typical experiment, all the peptides encompassing the TrpM (namely, peptides 58, 59, L15P, and C8) completely blocked antibody binding to L36I although control peptides 60 and $C8_{scr}$ did not, thus clearly indicating that the L36I-reactive antibodies present in all 4 Qβ-59 immunized cats were specific for the TrpM. This conclusion was then substantiated by producing a four-branched tetrameric analogue of peptide C8 (MAP-C8; Fig. 1) and using it to test the immune sera in ELISA. Indeed, all Qβ-59 immune sera, but not control sera, reacted with MAP-C8 (Table 1).

Fine mapping of the TrpM B epitope recognized by peptide 59-immunized cats

The above results had shown that the sera of Qβ-59-immunized cats specifically reacted with a sequence reproducing the TrpM of FIV TM. The reaction occurred with

Table 1
Summary of the reactivity exhibited by Qβ-59-immunized and FIV-infected cats against the synthetic peptides included in the study, as determined in direct ELISA tests

Peptide used	Serum from cats[a]		
	Immunized with coupled Qβ-59	Immunized with uncoupled Qβ and 59	FIV-infected
59	0/4[b]	0/3	0/24
58	0/4	0/3	0/24
60	0/4	0/3	0/24
L361	4/4	0/3	14/24
E29K	0/4	0/3	14/24
L15P	0/4	0/3	0/24
C8	0/4	0/3	0/24
Map-C8	4/4	0/3	0/24

[a] Preimmune sera consistently negative against all peptides.
[b] Positive/tested.

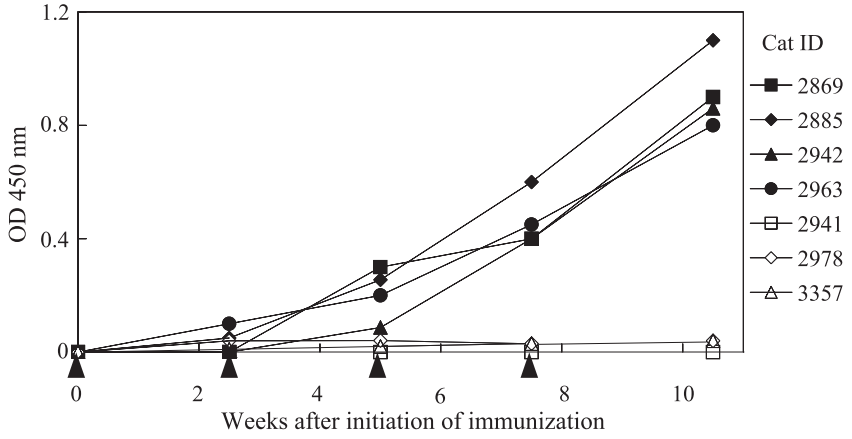

Fig. 3. TM L36I peptide antibody reactivity detected in sera from cats immunized with Qβ-59 or a mixture of uncoupled Qβ and peptide 59. Sera were obtained from cats immunized with either Qβ-59 (solid labels) or a mixture of uncoupled Qβ and peptide 59 (empty labels) at the time of each immunization (arrowheads) and 3 weeks after, diluted 1:100 and analyzed for L36I binding antibody on L36I-coated microwells by ELISA. Values, expressed as optical density (OD), are the means of duplicates that did not differ by more than 10%.

both large and short peptides, provided that the latter were in solution (not plastic-bound). It was therefore of interest to obtain a better definition of the precise epitope(s) involved. We thus investigated how a series of analogues of peptide C8, each having a different amino acid position substituted with alanine, behaved in binding competition with peptide L36I. As shown in Fig. 5, the analogues having an Ala substituted for the Trp at position 770, the Trp at position 773, or the valine at position 774 exhibited a markedly reduced ability to compete, essentially behaving as control $C8_{scr}$, whereas all others competed as effectively as the unsubstituted peptide C8. Furthermore, the competition profile was similar irrespective of the immune serum used, showing that the epitope recognized did not vary in individual animals.

Lack of seroreactivity of the TrpM in FIV virions and during FIV infection

The above results had clearly indicated that the sequence corresponding to the TrpM of FIV TM contained at least one potential B epitope. It was therefore important to ascertain whether the antipeptide 59 antibodies produced by immunizing cats with Qβ-59 bound FIV virions. No evidence of such reactivity was detected in ELISA tests using either intact or sonicated gradient-purified whole FIV virions as antigens, as well as in immunoblots under denaturing conditions (data not shown). Also, although adsorption of FIV-infected cat sera with purified virus and especially cell-adsorbed virus, but not with cells alone, removed a measurable amount of reactivity to peptide

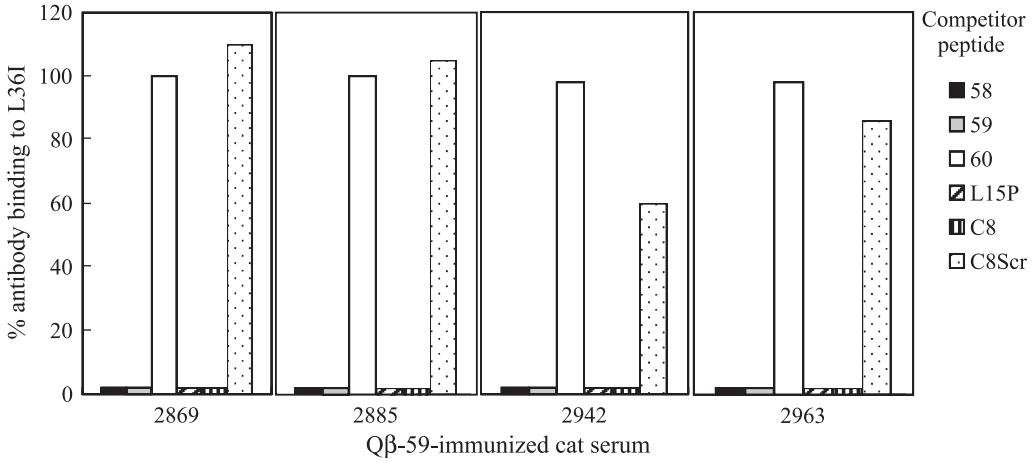

Fig. 4. Ability of selected peptides to compete with the anti-L36I peptide sera binding reactivity of individual Qβ-59-immunized cat sera. Sera diluted 1:100 (100 μl) were incubated 30 min at room temperature with the indicated competitor peptides (10 μg/ml, final concentration) or diluent alone and then tested for binding to peptide L36I-coated ELISA microwells. Binding was calculated using the formula: mean OD obtained with serum preincubated with competitor peptide/mean OD obtained with serum preincubated with diluent alone ×100.

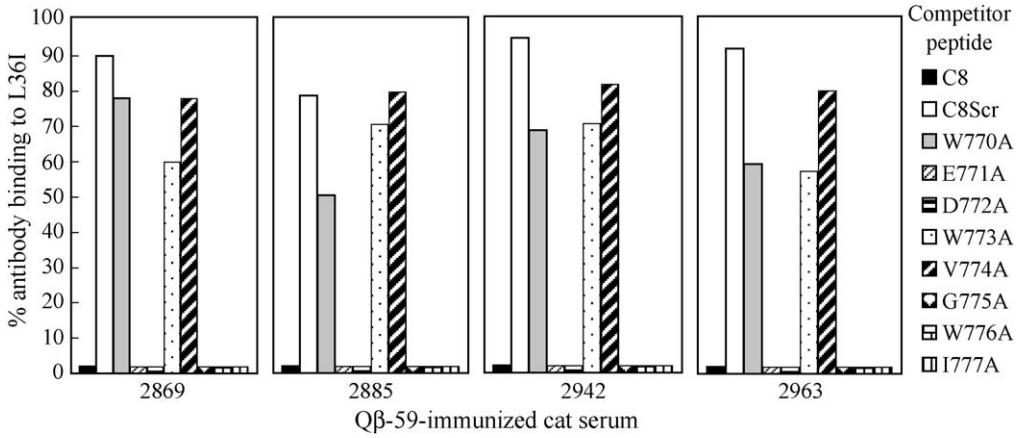

Fig. 5. Alanine scanning analysis of the B epitope recognized by Qβ-59-immunized cats. A series of analogues of peptide C8, each having a different amino acid position substituted with an Ala, were analyzed for their ability to compete with the binding reactivity to peptide L36I-coated ELISA microwells exactly as described in Fig. 4.

L36I, the same procedures failed to remove L36I reactivity from the Qβ-59-immunized cat sera (Fig. 6). Finally, FIV neutralizing activity in Qβ-59 immune cat sera was also undetectable by sensitive assays using either a recent virus isolate (FIV-M2) or a tissue culture-adapted strain (FIV-Pet) (data not shown).

In complementary experiments, we also examined whether the TrpM could evoke a humoral immune response during the course of FIV infection. This was done by testing sera of SPF cats at various times of infection with two strains of FIV against the entire set of peptides used in the study by ELISA. Several such sera reacted with peptides L36I and L29K, often at substantial levels, but none reacted with any of the other peptides (Table 1). Importantly, infected cat sera failed to react with any of the test peptides, including MAP-C8, that were instead recognized by Qβ-59-immunized cat sera. This makes it highly unlikely that some of the infected cat sera reactivity against peptide L36I was directed to the TrpM. This was, however, further explored by examining whether the binding of selected infected cat sera to peptide L36I could be competed with the use of peptides. As shown by Fig. 7, the only peptide other than the homologous peptide that consistently proved capable of competing in these assays was peptide E29K, that is, a peptide that overlapped peptide L36I but lacked the TrpM. The TrpM-containing peptides 59, L15P, and C8 were instead completely ineffective, although peptide 58 showed marginal competing ability, if any, possibly because of the N-terminal overlap with peptide L36I. It was therefore concluded that the antipeptide L36I reactivity observed in

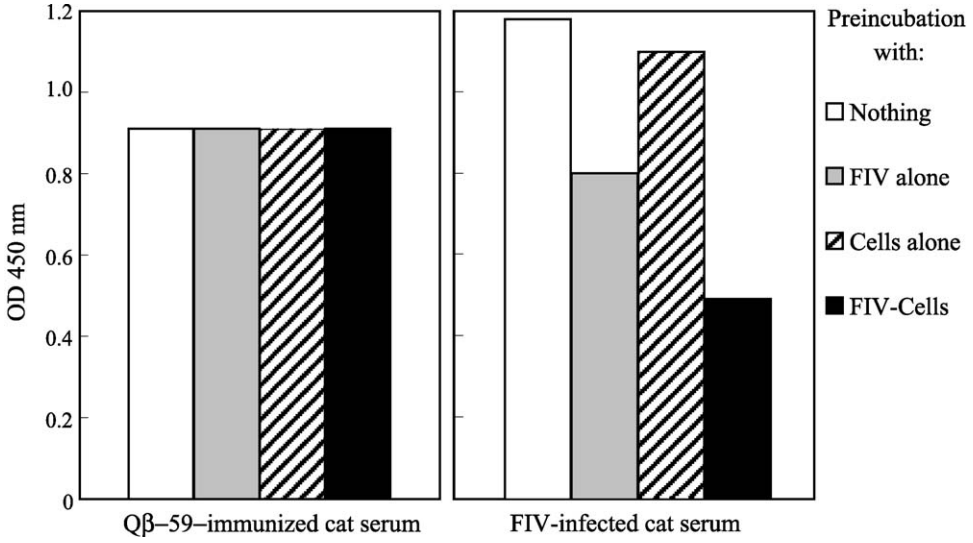

Fig. 6. Ability of FIV virions, substrate cells, or virus–cell mixtures to remove peptide L36I binding activity from the sera of Qβ-59-immunized and FIV-infected cats. Pooled FIV-infected or Qβ-59-immunized cat sera were diluted 1:100 and preincubated with virions (5 μg of FIV proteins), viable cells alone (5×10^5), and virions and cells (5 μg of FIV proteins with 5×10^5 cells) or diluent alone at 4 °C for 1 h, followed by 1 h at 37 °C. Adsorbed cat sera were centrifuged at $600 \times g$ for 15 min (to remove cells) followed by $20\,000 \times g$ for 90 min (to remove virions) and tested for binding activity to peptide L36I-coated ELISA microwells.

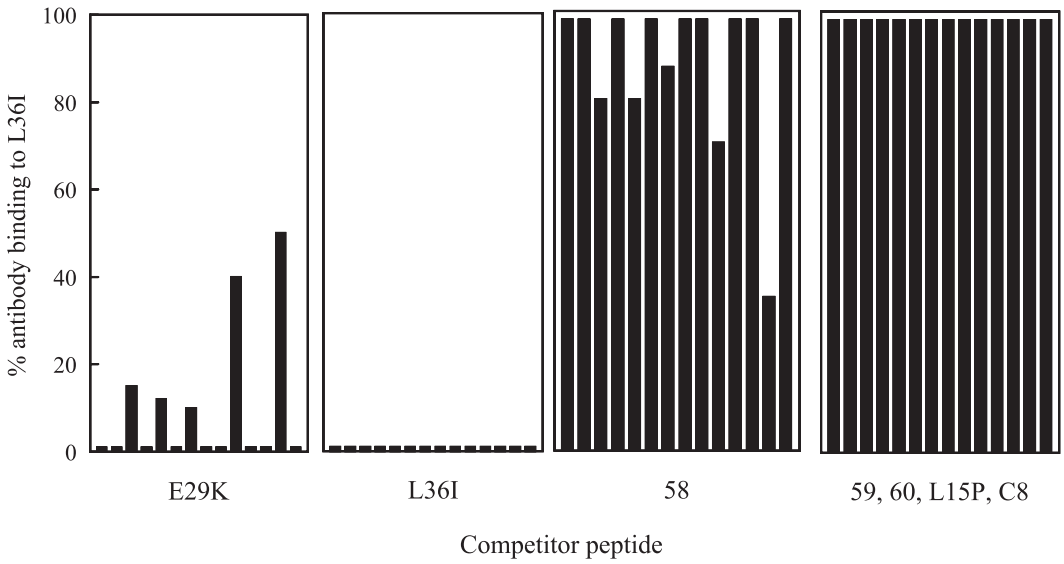

Fig. 7. Ability of selected peptides to compete with anti-L36I antibody binding reactivity of FIV-infected cat sera. Ten FIV-Pet-infected and 4 FIV-M2-infected cat sera that had shown anti-L36I reactivity in the experiment in Table 1 were diluted 1:100 (100 μl), incubated 30 min at room temperature with the indicated peptides (10 μg/ml, final concentration) or with diluent alone, and then tested for binding activity to peptide L36I-coated ELISA microwells. Results are expressed as in Fig. 4.

FIV-infected cat sera was solely directed to regions of the peptide other than the TrpM, and that the potential B epitope that had been detected in the sequence corresponding to the TrpM of FIV by immunizing cats with Qβ-59 could not be shown in the context of intact FIV virions.

Discussion

In recent years, the C-terminal half of the ectodomain of the HIV-1 TM glycoprotein has attracted considerable attention as a structure onto which antiviral drugs that block virus entry into cells can be modeled or targeted. Indeed, one such HIV-1 fusion inhibitor (T20 or enfuvirtide) is already in clinical use, and others are currently under active development (Doms and Moore, 2000; Jiang et al., 2002; Kilby et al., 1998; LaBranche et al., 2001). Moreover, at least two broadly reactive HIV-1 neutralizing monoclonal antibodies hitherto developed have been found to recognize amino acid sequences belonging to such a domain, and this has stimulated studies on the possibility of developing immunoprophylactic and immunotherapeutic agents targeted to the same region (Marusic et al., 2001; McGaughey et al., 2003; Muster et al., 1993; Parker et al., 2001; Xiao et al., 2001; Zwick et al., 2001).

FIV shares numerous biological and pathogenetic properties with HIV and is therefore an advantageous small animal model for exploring the feasibility of novel therapeutic and vaccinal strategies (Pedersen et al., 1987; reviewed in Elder et al., 1998; Overbaugh et al., 2001; Willett et al., 1997). In particular, the envelope glycoproteins of FIV and HIV exhibit common structural frameworks and seem to play similar roles in cell entry (de Parseval and Elder, 2001; Wyatt and Sodroski, 1998). In the present study, we analyzed the serological properties of the TrpM located in the C-terminal part of the ectodomain of the TM glycoprotein of FIV, in proximity of the transmembrane segment of the molecule. In analogy to what was observed for the corresponding zone of HIV-1 (Muñoz-Barroso et al., 1999; Salzwedel et al., 1999), it has been shown that the Trp residues present in the TrpM of FIV need to be conserved for efficient virus entry into cells (Giannecchini et al., 2004). Furthermore, this tract of the TM of FIV has been successfully used to model powerful inhibitory peptides, one of which (peptide C8) is only eight amino acids long and hence contains the TrpM alone (Giannecchini et al., 2003). It was therefore logical to investigate whether the TrpM of FIV might also represent a suitable target for immune intervention.

We started by ascertaining whether in the FIV TrpM contains potential epitopes capable of evoking an antibody response in cats. This was achieved by immunizing with the TrpM-containing peptide 59 covalently linked to recombinant Qβ VLP. Such a 20-mer peptide appeared particularly suitable because (i) its small size limited the number of possible non-TrpM epitopes; (ii) it had previously been shown to inhibit FIV replication in vitro (Lombardi et al., 1996), thus indicating that its TrpM, that is, the segment of the peptide responsible for such activity (Giannecchini et al., 2003), was in a biologically active conformation; and (iii) the presence in it of short amino acid tails at both sides of the TrpM was likely to permit its coupling without much distortion of the motif. Coupling the peptide to VLP was considered necessary because, in previous studies, uncoupled peptide 59 had failed to generate antibodies when administered to mice in Freund's complete adjuvant (Massi

et al., 1997). VLP permit to express epitopes as highly repetitive and organized arrays, and this has been shown to turn them into strong B cell activation stimuli (Jegerlehner et al., 2002a). Indeed, VLP have been successfully used to achieve potent B cell responses with poorly immunogenic peptides in the absence of adjuvants (Fehr et al., 1998; Jegerlehner et al., 2002b; Pumpens et al., 1995). The advantages of using Qβ VLP to this purpose have been recently illustrated (Jegerlehner et al., 2002a).

The immunization procedure that was chosen proved effective because all four cats given Qβ-59, but none of those who received a mixture of uncoupled Qβ VLP and peptide 59, developed antipeptide antibodies, which proved specific for the TrpM in extensive binding and binding competition assays. Notably, TrpM-specific antibodies were revealed by a 36-mer TrpM-containing peptide in binding assays, but not by four much shorter (8- to 20-mer) peptides that also contained the TrpM. However, all of the latter, including peptide C8 that consisted of the TrpM alone, were effective in inhibiting the binding of peptide 59-specific antibodies to the 36-mer peptide, thus showing that the epitope(s) involved belonged to the TrpM sequence. Although alternative explanations remain possible, it is likely that short peptides lost reactivity after having adhered to plastic because the epitope recognized was conformation-dependent (Joyce et al., 2002). Indeed, all positive sera reacted against MAP-C8, most likely because at least one peptide chain in this tetrameric analogue of peptide C8 remained available for antibody–antigen reaction (Caponi et al., 1995). In keeping with this possibility, alanine scanning of peptide C8 showed that the sera of all four Qβ-59 immunized cats reacted with a same epitope and that this was composed of three amino acids, two of which are nonadjacent. Interestingly, two of these were Trp, that is, residues previously shown to play a key role in the function(s) the TrpM exerts in cell entry (Giannecchini et al., 2004). In addition, the Trp residues have been found to determine the α-helix conformation the inhibitory peptide C8 has when it is dissolved in physicochemical conditions compatible with biological environments (Giannecchini et al., 2003). It is also noteworthy that the three amino acids that make up the epitope are closely associated and lye on the hydrophobic face of the helical wheel projection of the TrpM (Fig. 1). In the solid-phase context of direct ELISA, particularly in short peptides, this face is likely to interact directly with the plastic support, thus becoming unavailable for antibody binding.

Anti-TrpM sera, together with the panel of TM-derived peptides used for their characterization, were then exploited to investigate whether the TrpM is serologically active in the context of whole FIV virions and in the course of FIV infection. None of several methods used demonstrated binding of the anti-TrpM antisera to intact, sonicated, or cell-adsorbed FIV virions. Furthermore, the antisera failed to block infectivity of FIV in sensitive neutralization tests, no matter whether the virus used was of recent isolation or tissue culture-adapted. To sum up, all of the approaches used pointed to the conclusion that the TrpM is not available to antibodies on FIV virions, not even when these are interacting or have interacted with the cell surface. At least in HIV-1, such interaction has been shown to uncover novel epitopes not expressed by unadsorbed virions and to bring about conformational changes of the glycoprotein complex that ultimately lead to cell entry (Doms and Moore, 2000; Golding et al., 2002). In addition, we found no evidence that anti-TrpM antibodies are produced in the course of FIV infection of cats. In fact, none of the sera taken from numerous FIV-infected cats exhibited antibodies that bound the TrpM or competed with the anti-TrpM sera elicited with Qβ-59 in binding to such a motif. Although previous studies have used synthetic peptides to dissect the humoral response of FIV-infected cats (Avrameas et al., 1993; de Ronde et al., 1993; Pancino et al., 1993; Richardson et al., 1998), whether the TrpM is immunogenic in the course of FIV infection has not yet been specifically addressed. In one such study, peptides containing different segments of the TrpM, as well as short upstream and downstream sequences, were found to react with variable proportions of infected cat sera, but no attempts were made to exactly identify the epitopes involved (Avrameas et al., 1993). In HIV-1, TrpM-containing peptides of various lengths have been shown to react with highly variable proportions of infected human sera; however, the consensus is that the TrpM is poorly immunogenic, if at all. This is in line with the failure to elicit HIV-1 immune responses in recent attempts using immunogens modeling this, nearby or adjacent regions (Calarota et al., 1996; Conley et al., 1994; Muster et al., 1993; Xiao et al., 2001; Zwick et al., 2001).

According to current models, the TrpM of HIV-1 is highly flexible and it undergoes sequential conformation transitions from a closed turn to an extended arrangement and finally to an amphipathic α-helical structure as a result of TM activation following virus interaction with susceptible cells. The latter conformation would then be suitable for intimate and concomitant interaction with the viral and the cell membrane (Barbato et al., 2003), thus allowing Trp residues, with their bulky indole side chains, to destabilize both membranes and drive their fusion alone or in synergy with the fusion peptide (Melikyan et al., 2000; Peisajovich and Shai, 2003; Schibli et al., 2001; Suárez et al., 2000). It seems therefore possible that the conformation-dependent B epitope we have demonstrated in the TrpM of FIV by immunizing cats with Qβ-59 is available only when the TrpM has an α-helical conformation. This is when the viral and the cell membrane are too close to each other to be accessible to antibodies (Finnegan et al., 2001; Giannecchini et al., 2002), thus explaining why we were unable to demonstrate such an epitope on virions, no matter whether free or cell-associated. On the other hand, the flexible nature of the TrpM might render it a poor immunogen during FIV infection, thus explaining why it was found to be completely serologically silent in infected cats. In any case, there would

appear to be little hope that the TrpM of FIV can serve as an easy target for antibody-based antiviral strategies. It should, however, be noted that the implications of these findings cannot be directly extrapolated to HIV-1 without further investigation, because the TrpM of this virus differs from the TrpM of FIV under several regards, including number and spacing of Trp residues, that might bring about differences in their immunological behavior.

Materials and methods

Animals, cells, and viruses

SPF female cats were purchased from Iffa Credo (L'Arbresle, France) and immunized when 7–12 months old. Animals were housed in our climatized animal facility under conditions required by the European Community Law. The viruses used were FIV-Pet and FIV-M2. The former was a tissue culture-adapted strain of clade A and was obtained from chronically infected FL4 cells (kindly provided by Dr. J. Yamamoto, Gainesville, FL), while the latter was a recent isolate of clade B and was grown in MBM cells. MBM cells are an interleukin (IL)-2-dependent line of T lymphocytes originally established from the peripheral blood mononuclear cells of an FIV- and feline leukemia virus-negative cat (Matteucci et al., 1995). They are routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5 μg of concanavalin A and 20 U of IL-2 per ml.

Peptide synthesis

Synthetic peptides (Fig. 1) were prepared manually by a conventional solid-phase strategy, as described previously (D'Ursi et al., 2003). Crude peptides were purified by semi-preparative reverse-phase high-pressure liquid chromatography (HPLC) to a purity greater than 95% and lyophilized. Final products were characterized by analytical HPLC and electrospray mass spectrometry (ESI-MS). MAP-C8 was synthesized starting from a branched poly-lysine central core, prepared coupling on the resin two levels of Fmoc-Lys(Fmoc)-OH onto a β-alanine residue, as shown in Fig. 1. The crude peptide was lyophilized twice, characterized by ESI-MS, and used without further purification.

Expression and purification of Qβ

Capsids of phage Qβ were expressed using the expression vector pQβ10 and purified as described (Cielens et al., 2000; Kozlovska et al., 1993). In brief, *E. coli* lysates containing the expressed coat protein were cleared by centrifugation. Proteins from the supernatant were fractionated by ammonium sulfate precipitation (at final concentrations of 20% and 40% saturation, sequentially). The precipitated capsids were resuspended in a minimal volume of gel filtration buffer containing 20 mM Tris–HCl, pH 7.8, 5 mM EDTA, and 150 mM NaCl and purified over a Sepharose CL-4B column (Amersham Bioscience, Uppsala, Sweden). Eluted capsids were precipitated with polyethylene glycol-6000 at 13.3% saturation and repurified on a Sepharose CL-4B column. Capsids present in the peak fractions were precipitated with ammonium sulfate at 60% saturation. Sedimented VLP were resuspended in gel filtration buffer and loaded onto a Sepharose CL-6B column (Amersham Bioscience). Fractions containing the Qβ were pooled, concentrated by ammonium sulfate precipitation, and dialyzed against 20 mM HEPES, 150 mM NaCl, pH 7.4.

Coupling of peptide 59 to Qβ

Qβ VLP (2 mg/ml) were mixed with a fivefold molar excess of the cross-linker succinimidyl-6-[β-maleimido-propionamido]hexanoate (Pierce, Rockford, USA) and incubated for 30 min at room temperature. The reaction was performed in 20 mM HEPES, 150 mM NaCl, pH 7.2. Free, unreacted cross-linker was removed by dialysis against 50 mM phosphate, 150 mM NaCl, pH 6.5 (coupling buffer), using SnakeSkin® dialysis membranes (Pierce) with a cutoff of 3.5 kDa. Derivatized Qβ VLP was mixed with a half molar equivalent (based on the concentration of the subunits) of peptide 59 containing a C-terminal cysteine, dissolved in dimethyl sulfoxide (DMSO), and incubated at 15 °C for 2 h to obtain Qβ-59. Uncoupled peptide was removed by dialysis with Slide-A-Lyzer® Cassettes, cutoff 10 kDa (Pierce), against coupling buffer overnight at 4 °C. Coupling efficiency was evaluated by SDS-PAGE analysis and density measurement of the uncoupled Qβ band versus the heavier peptide-coupled one (Fig. 2).

Because of its high content in hydrophobic residues, peptide 59 tended to precipitate out of aqueous solution. For this reason, DMSO was used to dissolve the peptide, and it was present in the buffer during coupling at a maximal concentration of 10%. Nevertheless, peptide 59 could be found also as a precipitate at the end of the coupling reaction (not shown). This was probably the reason why, in repeated attempts, coupling efficiency between Qβ subunits and peptide 59, expressed as ratio between the densities of the coupled and uncoupled Qβ bands, was never higher than 20%. For the present study, we used antigen derived from a same coupling reaction, in which the proportion of coupled VLP subunits was approximately 17% (Fig. 2). Although suboptimal, this extent of coupling was considered sufficient for a proof-of-concept study.

Immunization schedule

Four SPF cats were inoculated subcutaneously with 78 μg of coupled Qβ-59 carrying total 2.5 μg of peptide 59, while three controls were inoculated with a mixture of 78 μg of Qβ and 2.5 μg of uncoupled peptide. Four doses were administered at 2.5-week intervals, and serum was obtained from

each cat before the first inoculum, at the time of every immunizing dose, and 3 weeks after the last inoculum. All procedures were carried out under light anesthesia.

Antibody binding, binding competition, and neutralization assays

Total cat immunoglobulin G (IgG) to gradient-purified and sonicated whole FIV and TM peptides were measured by ELISA, as previously described (Mazzetti et al., 1999). Briefly, ELISA microwells were coated overnight with 100 μl of 1 μg/ml of either whole FIV or 10 μg/ml of the synthetic peptides in carbonate buffer, pH 9.6. After a postcoating step with skim milk, serially diluted sera were added to the plates in duplicate. Bound IgG was revealed with a mouse anti-cat IgG antibody followed by a goat anti-mouse peroxidase conjugate. Virus neutralization tests were performed against 10 $TCID_{50}$ of FIV by using MBM cells as indicator cells and quantitation of reverse transcriptase activity in the supernatant as an end point read-out, exactly as described (Giannecchini et al., 2002).

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