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Collection of phage–peptide probes for HIV-1 immunodominant loop-epitope

Yadira Palacios-Rodríguez [a], Tatiana Gazarian [b], Merrill Rowley [c], Abraham Majluf-Cruz [d], Karlen Gazarian [a,*]

[a] Department of Molecular Biology and Biotechnology of Institute of Biomedical Research, Mexico City, Mexico
[b] Department of Public Health of Faculty of Medicine, Mexican National Autonomous University, Mexico City, Mexico
[c] Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia
[d] General Hospital No. 1 Gabriel Mancera, IMSS, Mexico

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Abstract

Early diagnosis and prevention of human immunodeficiency virus type-1 (HIV-1) infection, which remains a serious public health threat, is inhibited by the lack of reagents that elicit antiviral responses in the immune system. To create mimotopes (peptide models of epitopes) of the most immunodominant epitope, CSGKLIC, that occurs as a loop on the envelope gp41 glycoprotein and is a key participant in infection, we used phage-display technology involving biopanning of large random libraries with IgG of HIV-1-infected patients. Under the conditions used, library screening with IgG from patient serum was directed to the CSGKLIC epitope. Three rounds of selection converted a 12 mer library of 10^9 sequences into a population in which up to 79% of phage bore a family of CxxKxxC sequences ("x" designates a non-epitope amino acid). Twenty-one phage clones displaying the most frequently selected peptides were obtained and were shown to display the principal structural (sequence and conformational), antigenic and immunogenic features of the HIV-1 immunodominant loop-epitope. Notably, when the mixture of the phage mimotopes was injected into mice, it induced 2- to 3-fold higher titers of antibody to the HIV-1 epitope than could be induced from individual mimotopes. The described approach could be applicable for accurately reproducing HIV-1 epitope structural and immunological patterns by generation of specialized viral epitope libraries for use in diagnosis and therapy.
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1. Introduction

Presently, HIV continues to spread over the continents, and has become the most dangerous slowly progressing pandemic threat (Rambaut et al., 2004; UNAIDS, 2005). No prevention has been found so far (McMichael, 2006) because the virus has the unique property of escaping both natural and laboratory-created prevention and therapeutic measures (Altman and Feinberg, 2004). Of primary concern in the efforts to developing effective protection against HIV is the lack of peptide substitutes of immunodominant epitopes to reproduce the crucial antigenic and immunogenic viral properties in experiments and trials. One approach has been the use of sequences of HIV epitopes to synthesize peptides using chemical methods. In fact, synthetic peptides representing viral epitopes are widely produced and used in diagnosis (Gnann et al., 1987) and in experiments on inhibition of viral entry into the target cell (Eckert and Kim, 2001). The major deficiency of these peptides is that their conformations are not stable, so they are non-homogeneous, tend to form aggregates, and, in general, do not display the natural epitope structure–function pattern correctly (Oldstone et al., 1991; Pan Chan Du et al., 2002). Another approach, is to allow the anti-HIV-1 antibody itself to recognize and select perfect peptides from vast phage epitope libraries (Parmley and Smith, 1988; Scott and Smith, 1990; Smith and Petrenko, 1997; see reviews Deroo and Muller, 2001; Gazarian,

* Corresponding author. Department of Molecular Biology and Biotechnology, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM), Ciudad Universitaria, Circuito Escolar S/N, C.P. 04510 México D.F., México. Tel.: +52 55 5 622 9206; fax: +52 55 5 622 9212/550 0048.
E-mail address: karlen@servidor.unam.mx (K. Gazarian).

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2005), displaying up to 10^{10} linear or constrained peptide sequences (Scholle et al., 2005). Currently, phage display is a rapidly progressing biotechnology that allows presentation and reproduction of diverse panels of engineered peptides and proteins. Their screening (*biopanning*: Parmley and Smith, 1988) retrieves collections of peptide substitutes of ligands of functional proteins (Kay and Hamilton, 2001), antibodies in particular, and peptide probes for detection of biological treatment agents (Petrenko and Vodyanoy, 2003). The methodology combines many technical elements of current protein and peptide expression and modification methods with simple microbiological procedure of fast amplification of hundreds of selected peptides.

Despite the advantages that the phage display offers for many systems, there is still a dearth of publications dealing with the selection of peptides for HIV-1 (Scala et al., 1999; Enshell-Seijffers et al., 2001). One reason for this is that HIV-positive serum, which is rich in virus-specific antibodies useful for isolation of valuable peptides, is inconvenient for successful selection due to the heterogeneity and variability of the antibody species in individuals, so that peptides for epitopes of special interest are only isolated by chance. The purpose of our experiments was to develop conditions for the high-yield selection of peptides for one particular epitope, the HIV-1 immunodominant (ID) epitope $_{603}$CSGKLIC$_{609}$ presented as a loop on the apex of the gp41 protein domain interacting tightly with gp120, with which the gp41 forms the heterotrimeric complex involved in the infection process.

In earlier attempts to isolate HIV-1-specific peptides, serum samples of two HIV-1(+) non-symptomatic patients were used to screen m13 gp8 libraries but no gp41 ID mimotope containing two cysteines to adopt the loop configuration similar to the epitope were isolated (Scala et al., 1999). In a second study, the screening of a 12 mer constrained library yielded three 5 mer peptides with CSGKLIC-like sequences (Enshell-Seijffers et al., 2001; peptides shorter than are designed for the library arise sometimes during construction as a result of deletions). To improve the results of mimotopes selection for this particular epitope, we performed a total of 10 biopanning experiments involving different gp3 peptide libraries and a panel of sera from patients with disease progression which at the time of the study initiated the multidrug antiretroviral therapy (HAART: highly active antiretroviral therapy); we determined the library parameters and the titer of serum antibody to the epitope ensuring large-scale and highly reproducible selection of peptides to the gp41 epitope.

2. Materials and methods

2.1. Serum and IgG

The donors of HIV-1-infected serum were four patients (designated Pt: 1 to 4) of the "Hospital General No. 1 Gabriel Mancera, IMSS", Mexico City, infected with B-subtype HIV-1 circulating in Mexico and were at the stage of disease progression requiring HAART. Viral load (RNA copies/ml) and CD4 counts (cells/mm^3) prior to and after 12–15 weeks of the therapy were as follows: Pt 1 = 30,400/277 and 51,600/315; Pt 2 = 24, 700/267 and 20,300/239; Pt 3 = 116,000/26 and 5840/136; Pt 4 = 750,000/42 and 119,000/169. The drug combinations for HAART were: Pt 1 and 2: nucleoside ZDV (zidovudine) and ddC (zalcitabine) + non-nucleoside EFV (efavirenz); Pt 3 and Pt 4, ZDV, ddC with protease inhibitor NFV (nelfinavir). Serum was prepared from the blood drawn in the hospital and stored at −20 °C. The IgG fraction was isolated by single-step affinity purification with protein G-Agarose (Life Technologies, USA) (Gazarian et al., 2000) and stored in aliquots at −20 °C (yield of IgG was 80–85% taking its amount in serum as 8–10 mg/ml). Protein in the preparations was quantified by the Bradford assay and could include some non-IgG serum components.

2.2. Overlapping synthetic peptides

Peptides (6354) VLAVERYLKDQQLLG, (6355) ERYLKD QQLLGFWGC, (6356) KDQQLLGFWGCSGKL, (6357) LLG FWGCSGKLICTT, (6358) WGCSGKLICTTTVPW), (6359) GKLICTTTVPWNASW, were from the NIH AIDS Research and Reference Reagent Program.

2.3. Phage-display libraries

Peptide libraries, 12 mer linear, and 7 mer linear and constrained, were from New England BioLabs Inc. (Beverly, MA, USA). In each of them, random peptides (approx. 2.7×10^9 electroporated sequences) are fused to a minor coat protein (gp3) of m13 phage and expressed at its N-terminus separated by a Gly-Gly-Gly-Ser spacer. The libraries were amplified once to have 55 to 200 copies of each sequence per 10 µl, the amount used in single biopanning experiment.

2.4. Peptide selection and analysis

A standard biopanning procedure (Smith and Scott, 1993; Dower and Cwirla, 1994; Smith and Petrenko, 1997) was used with minor adjustments (Gazarian et al., 2001). Briefly, two wells in a 96-well polystyrene microtiter plate (Immulon 4 flat-bottom plates, Dynatech Lab Inc., USA) were coated with affinity purified IgG from each patient's serum. For this, one well was filled with 15 µg, the second with 7.5 µg of IgG, each in 100 µl of phosphate-buffered saline (PBS), and the plates were incubated overnight at 4 °C with gentle rocking. Unbound IgG was removed and the wells were washed 6 times with PBS-T (PBS–0.1% Tween 20) and blocked 1h at 4 °C with blocking buffer (PBS–1% BSA), followed by 5 consecutive washing steps using PBS-T. For affinity selection, 10 µl of the library (2×10^{10} plaque-forming units, PFU) were added to 190 µl of PBS-T and the mixture was distributed in the wells coated with IgG (100 µl/well); the wells were incubated for 1 h at room temperature (RT) with gentle rocking to allow phage to bind. The unbound phage were pipetted out and wells were washed 10 times with PBS-T (RT). Bound phage were eluted from each well by stirring with 100 µl of elution buffer (0.1 N HCl–glycine, pH 2.2). Eluted phage (designated further as "eluate") from two wells were combined and quickly neutralized by the addition of 2 M Tris (pH 9.1).

Phage were quantified by titering on log-phase *Escherichia coli ER2738* (BioLabs) on LB plates and the eluate was amplified in 30 ml of 2× YT for 4 h. Cells were pelleted by centrifugation, PEG/NaCl (20%/40%) was added to supernatant (overnight at 4 °C), amplified phage pelleted by centrifugation (11,000 rpm, 10 min, 4 °C), resuspended in PBS and clarified by centrifugation (11,000 rpm, 2 min, 4 °C); the supernatant was transferred to a fresh centrifuge tube for the second phage precipitation with PEG/NaCl, as described. Phage collected after this step were titered, resuspended in PBS at concentration 10^{12}–10^{13} PFU/ml and used in the second round of affinity selection, as described above, using two wells with the same amounts of IgG. The third round of selection was done similarly, with the difference that the stringency conditions in the washing solution PBS-T were increased by using PBS–0.5% Tween 20 (instead of 0.1% used above). The third round eluate was titered, small portions ("samples") were plated, and individual plaques were picked up randomly and amplified 4.5 h in 3 ml of 2× YT. Single-stranded DNA prepared from each clone was sequenced and the amino acid sequence of peptides was deduced. The homology of the sequences was determined using Pileup–Tudos algorithm as previously (Gazarian et al., 2001). Reactivity of clones with IgG was assayed by ELISA. Microtitration wells (Nunc-Immuno Plate, MaxiSorp F96, NUNC Brand Products, Roskilde, Denmark) were coated by overnight incubation (4 °C) of phage suspension (100 µl, 1×10^9–1×10^{10} PFU in PBS–50 ng BSA).

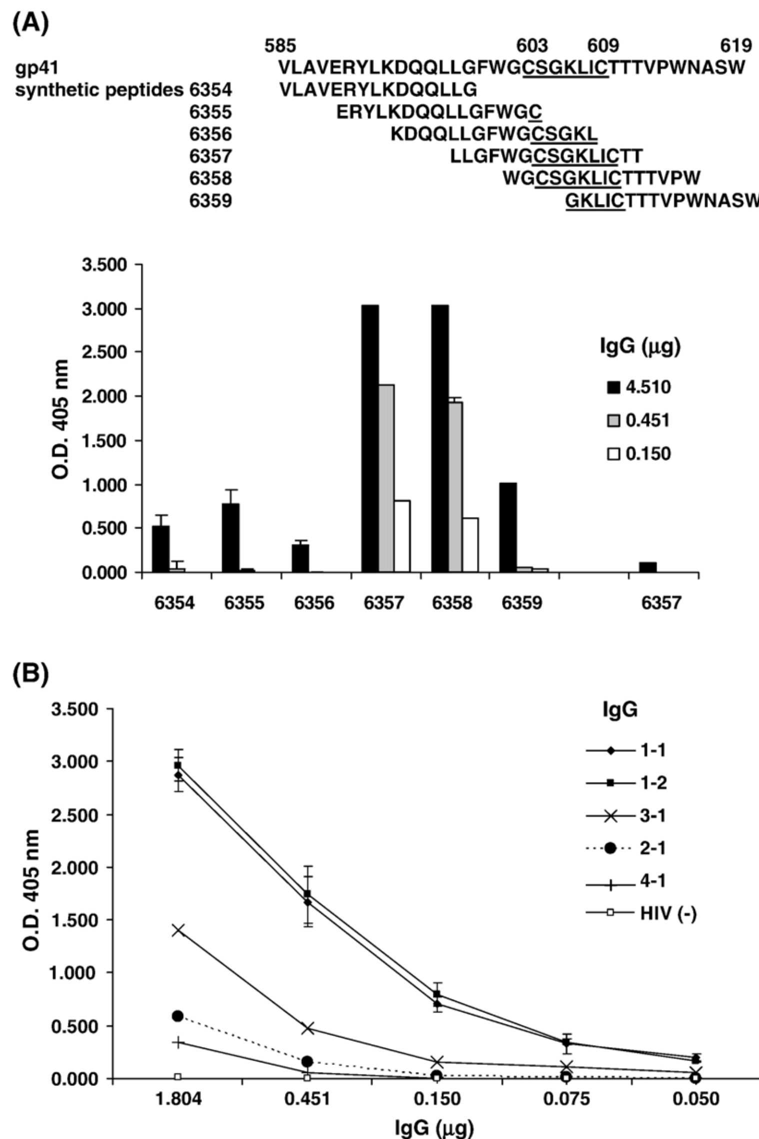

Fig. 1. Assessments of antibody levels to the gp41 ID epitope in IgG purified from sera of patients. (A) Determining the sequence in the gp41 ID domain that is recognized by Pt 1 IgG. Top: gp41 immunodominant amino acids (585–619) and the sequences of the overlapping synthetic peptides used in the ELISA. The loop-forming CSGKLIC sequence is shown underlined. Bottom: reactivity of different concentrations of pre-HAART Pt 1 IgG with the overlapping peptides. See Materials and Methods for ELISA conditions. (B) Titration of patient IgG for the Ab reactive with peptide 6357 (LLGFWGCSGKLICTT) used as epitope-representative antigen. The wells were coated with the peptide and incubated with different amounts of IgG of four different HIV-1(+) patients and of a HIV-1(−) blood donor as a control. In this figure and also throughout the text, the IgG preparations were given the following designations: 1–1, 2–1, 3–1, 4–1 are pre-HAART IgG of Pt 1, Pt 2, Pt 3, Pt 4, respectively; 1–2 designates Pt 1 4-week post-HAART IgG.

Wells with bound phage were washed 6 times with PBS–0.3% Tween 20, blocked for 1 h at 37 °C with PBS–1% BSA, incubated for 1 h at 37 °C with 5 mg/well (or as indicated in figure legends) IgG in PBS–0.2% Tween 20–0.2% BSA and washed with PBS–0.3% Tween 20. Bound Ab was detected using anti-human alkaline–phosphatase-conjugated Ab (Zymed Laboratories Inc., USA) diluted 1:1000 and *p*-nitrophenyl phosphate substrate diluted in diethanolamine buffer. After incubation at 37 °C, absorbance was read at 405 nm in an automated reader.

Wells were coated with synthetic peptides prepared in 0.2 M pH 9.5 carbonate buffer, by overnight incubation (2 μg/100 μl/well) at 4 °C and assayed by ELISA as described above for phage. In competitive ELISA, synthetic peptide 6357 with the CSGKLIC sequence (see above) was added to parallel probes to test for its ability to compete with the mimotope for IgG.

2.5. Immunization

Female C57BL/6J mice (15–16 g), 3 to 4 weeks old, were immunized (Galfré et al., 1996; Gazarian et al., 2000) by three i.p. injections of 2.5×10^{11} PFU of phage and were bled 10 days after the 4th injection for serum preparation. Serum was either used freshly or kept at −20 °C.

3. Results

3.1. Assessment of serum antibody recognizing the gp41 CSGKLIC sequence

An attempt to select phage predominantly expressing peptides for single epitope, in this case the HIV-1 gp41 ID epitope, from a library of over 10^9 different sequences using patient serum is a “search for a needle in a molecular haystack” (Rodi and Makowski, 1999). Despite the fact that the antibody to this epitope is known to exist in serum of 90% or more of infected subjects (Gnann et al., 1987; Horal et al., 1991), its amount varies significantly, even being non-detectable with current methods in some HIV-1-infected individuals (Oldstone et al., 1991). To find out whether the selection of these peptides depends on the antibody titer, and to determine the amount of antibody required in IgG for successful selection, the antibody to the gp41 ID epitope CSGKLIC was titered in IgG preparations and the results compared with the yield of the epitope-like peptide sequences derived after screening of peptide libraries. Fig. 1A shows the results of the ELISA in which IgG purified from the serum of Pt 1 was allowed to react with overlapping synthetic peptides covering the gp41 CSGKLIC region aa585–aa619. Two peptides containing the complete CSGKLIC sequence were most reactive, of which peptide No. 6357 was used further as antigen representing the epitope to assess the antibody titers (Fig. 1B). The IgG of Pt 1 obtained before and then 4 weeks after the initiation of HAART showed the highest reactivity. The pre-HAART IgG of Pt 3 showed an intermediate level, and IgG of Pt 2 and Pt 4 were weak binders. These data indicated that HAART, which effectively reduces the viral load in patients (Coffin, 1995) and decreases levels of anti-HIV-1 antibody in serum (Morris et al., 2001), also led to significantly lower titers of the antibody reactive to gp41 ID epitope (peptide 6357); the effect was minimal in the Pt 1 (Fig. 1B) and much stronger in other patients (not shown).

3.2. Biopanning of libraries with IgG of patients and description of selected peptides

We used four pre-HAART IgG preparations with different titers of antibody to the gp41 ID-epitope (Fig. 1B), five preparations of IgG obtained after different periods of the HAART (not included in the Table 1), and IgG of two HIV-1-negative subjects, in total, 11 IgG preparations, to perform a series of biopanning experiments with three libraries, a 12 mer linear, a 7 mer linear, and a 7 mer constrained. First, the pre-HAART IgG of Pt 1, that contained the highest titer of antibody to the epitope, was used to test the two 7 mer libraries but no peptide with a similarity to the CSGKLIC epitope was found in the sequenced samples. However when the 12 mer library of similar complexity (see Materials and methods) was screened with IgG prepared prior to HAART, then after 4, 12 and 15 weeks of the HAART, various results were obtained. No CSGKLIC-similar sequences were found after panning on pooled control IgG or on IgG of following patients, Pt 1 after 12 weeks of HAART, Pt 3 after 4 and after 15 weeks of HAART. The pre-HAART Pt 2 and Pt 4 IgG preparations also were inefficient. The latter showed very low antibody titers by ELISA (Fig. 1B). In contrast, three IgG preparations, those of the Pt 1, prepared before and after 4 weeks of HAART, and the pre-HAART IgG from Pt 3, that showed high to modest reactivity with the synthetic CSGKLIC peptide by ELISA, selected peptides with the motif CxxKxxC (Fig. 2 and Table 1) reminiscent of the gp41 loop-epitope CSGKLIC (Fig. 2A). In Fig. 2B, a typical image of the selected phage–peptides is shown; the CxxKxxC part is presented in cyclic form, based on evidence that sequences with two cysteines displayed on phage tend to cyclize (Menendez et al., 2004). Fig. 2C describes the

Table 1
Dependence of selection of CxxKxxC peptides on titer of Ab in IgG reacting with synthetic peptide containing epitope CSGKLIC sequence

Patient	IgG [a]	HAART week	Peptides in sequenced sample from 3rd round			
			Number of peptides in sample	CxxKxxC total (%)/ unique (%) [b]	Non-CxxKxxC	IgG (μg)/ O.D. 0.5 [c]
Pt 1	1–1	0	33	26 (79)/13 (39)	7	0.075
	1–2	4	30	13 (43)/7 (23)	17	0.075
Pt 3	3–1	0	61	16 (26)/ 8 (13)	45	0.45

[a] The 3 (out of 9 IgG) that presented CxxKxxC peptides in the sequenced samples; the results of the biopanning with IgG, that did not present CxxKxxC, are not included.

[b] Number and percent of CxxKxxC peptides in sequenced samples: total (includes the repetitions)/unique sequences.

[c] Titer of the epitope-specific Ab in IgG that was sufficient to present CxxKxxC peptides; expressed as μg IgG giving in ELISA an absorbance of 0.5 with synthetic peptide containing the epitope CSGKLIC sequence.

structure of the collection of 21 individual mimotopes selected in the three independent experiments (Table 1). The pre-HAART IgG from Pt 1 (designated in this figure, in Table 1 and in the text “1–1”) was most efficient: 79% of the peptides in the sample contained the motif. The origins of the non-CxxKxxC peptides were not investigated. The 4-week post-HAART IgG from Pt 1 (designated “1–2”), had about two-fold lower ability to retrieve mimotopes (43%). Even less was the yield of mimotopes in the selection with pre-HAART IgG “3–1” from Pt 3 (26%). With the remaining IgG preparations (pre-HAART IgG from Pt 2 and Pt 4, 4-week post-HAART IgG of Pt 3, 12- and 15-week post-HAART IgG of Pt 1 and Pt 3, respectively, and 24-week post-HAART IgG of Pt 3) no motif-peptides were found, although some other interesting sequences were selected (not shown in Table 1).

Some peptides appeared in more than one copy in the same or, importantly, in two or three independent experiments (indicated inside the loops, Fig. 2C). The most frequently selected mimotopes were: No. 3 (selected in 12 copies: 3 by IgG “1–1”, 3 by IgG “1–2” and 6 by IgG “3–1”); No. 13 (3 copies selected by three IgG: “1–1”, “1–2” and “3–1”); No. 1 (5 copies, 4 selected by IgG “1–2” and 1 by IgG “3–1”); No. 4 (2 copies: 1 selected by IgG “1–1” and 1 by IgG “1–2”); No. 11 (appeared in 4 copies, 2 selected by IgG “1–1” and 2 by IgG “3–1”). The independent selection of same peptide is indicative of the high affinity of the selecting antibody to these peptides, but could be due to the advantages these peptides gave to the phage in the course of the infection and/or replication. Some characteristics of the selection of these epitope-mimics are noted below.

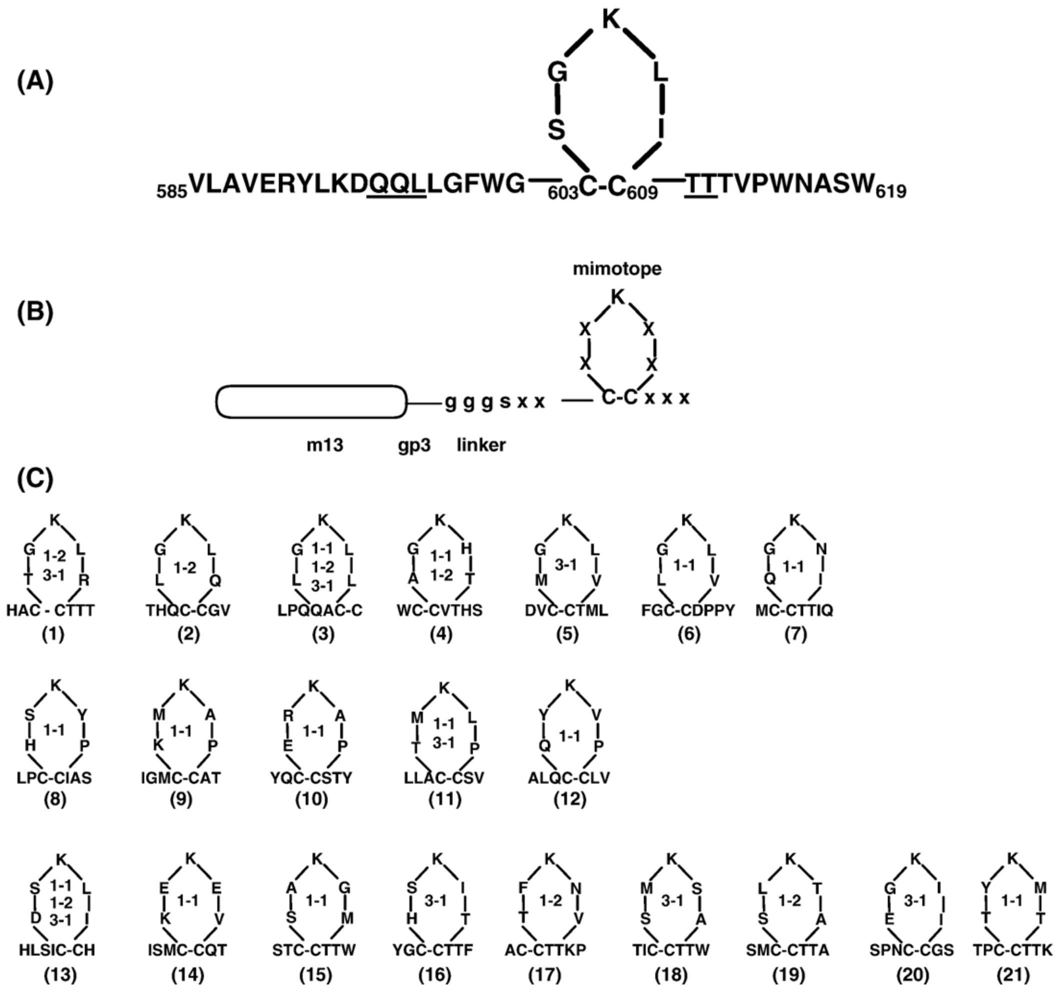

Fig. 2. Sequence data on the gp41 ID epitope region and on the selected mimotope collection. (A) The HIV-1 gp41 ID region encompassing the loop-forming CSGKLIC epitope sequence. Apart from the loop, the underlined residues were suggested to be part of the immunodominant epitope (Xu et al., 1991; Stigler et al., 1995). (B) Schematic representation of phage displayed 12 mer peptides with the CxxKxxC motif bound via the GGGS linker to gp3 N-terminus of the m13 phage (“x” are epitope positions Ser-Gly and Leu-Ileu (see “A”) which in mimotopes are occupied by other amino acids (shown below)). (C) Twenty-one consecutively numbered loop-mimotopes selected in three biopanning experiments by screening of the 12 mer library; inside each loop the IgG (1–1, 1–2 and 3–1) that selected the mimotope is indicated. (D) The homology relations of the epitope and the mimotope sequences shown by the Pileup–Tudos algorithm. By (*) we marked the sequence GTKLVC selected by Scala et al. (1999) (to which we added a cysteine in this homology analysis), and two sequences (CAGKLTC and CLGKMGC) selected by Enshell-Seijffers et al. (2001). The epitope sequences are: CSGKLIC (subtypes of HIV-1 A, B, C and F), CSGKHIC (subtypes D and G) and CSGKIIC (subtype E). Epitope CGSKLIC and mimotope CAGKHTC are in duplicate. The lengths of the bars reflect the relative levels of homology. In parenthesis is the mimotope number (as in Fig. 2C). Proline-containing peptides (Nos. 9–12).

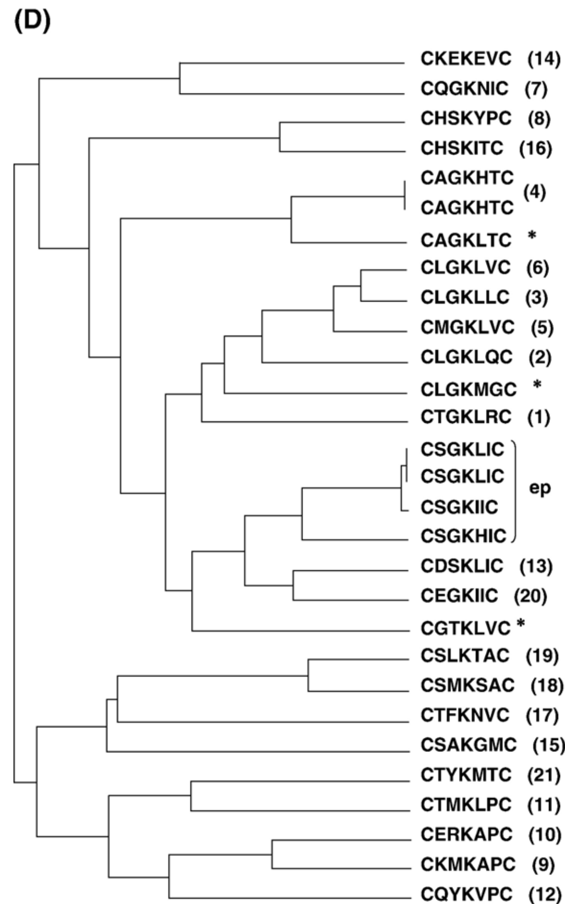

Fig. 2 (*continued*).

First, it is remarkable that constrained CxxKxxC sequences were found by the antibody in the non-constrained library of phage (see also Gazarian et al., 2003), in which the cysteine content is intentionally made about 10-fold lower than a random level, to decrease formation of Cys-Cys bridges between the peptide and the gp3 cysteines. Second, the higher efficiency of selection of epitope motifs shown by the pre-HAART IgG of Pt 1 as compared to that of the IgG from Pt 3 correlated with the higher titer of the antibody to the epitope in the IgG of Pt 1. Of the 9 HIV-1(+) IgG preparations used in the biopanning experiments, only three had antibody in amounts above the level required for the motif sequences to appear in samples (Table 1). Comparing the Ab titers in the IgG of the Pt 2, Pt 3 and Pt 4 (Fig. 1B) with their ability to select mimotopes (Table 1), one can see that IgG of Pt 3 contained this minimum amount of antibody for successful selection. From this we estimate that, to be efficient, IgG has to show an absorbance 0.5/0.45 μg of IgG. This is a threshold level of the epitope-specific Ab in IgG empirically estimated in the experiments that distinguished potent from non-potent IgG preparations regarding the ability to bring mimotopes in small samples of the screened library. The IgG from Pt 1 showed much higher levels of the antibody than is the threshold: O.D.=0.5/0.075 μg IgG, i.e. six-fold higher titer. From these data one may imply that the failure of 6 of the patient IgG preparations to select mimotopes was due to the lower antibody titers.

3.3. Structural relatedness of selected mimotopes to gp41 ID epitope

To reveal fine homology relations of the mimotopes with gp41 ID CSGKLIC epitope, we aligned their sequences using the Pileup–Tudos algorithm as in our previous study (Gazarian et al., 2001). The following sequences were included: the gp41 ID epitope sequences, CSGKLIC (subtypes A, B, C and F), CSGKHIC (subtypes D and G), CSGKIIC (subtype E); the 21 CxxKxxC mimotope sequences selected in this work (Fig. 2C); the three sequences selected previously (marked by asterisk), namely, GTKLVC isolated by Scala et al. (1999) that lacks one of the cysteines (the cysteine it contains we added for the homology analysis), and the CAGKLTC and CLGKMGC isolated by Enshell-Seijffers et al. (2001). All these were pooled and analyzed (Fig. 2D). The epitope sequence CSGKLIC and the mimotope sequence CAGKHTC were included in duplicate as internal controls of the alignment precision. The following were the results of the alignment: (i) subtypes A, B, C and F epitope sequences were aligned together and located in the center of the guide-tree (marked "ep"); above them one can see the mimotopes most homologous to the epitope sequences; (ii) in contrast, three proline-containing mimotopes (Nos. 9–11) that alter the epitope conformation lie together distantly from the epitope sequences; (iii) the two "best" mimotopes, No. 3 and No. 6, that were most frequently found (Fig. 2C) and most

reactive (Fig. 3A), were aligned together, and show the highest after the epitope sequences homology with each other. In general, we find that the alignments are in concordance with the structural and immunological characteristics of the selected collection.

3.4. Antigenic and immunogenic relatedness of selected peptide collection to the gp41 ID epitope

The antigenicity of mimotopes was determined by measuring their ability to bind in ELISA to the IgG used for their selection (Fig. 3A). In these assays, most of the CxxKxxC peptides bound to the selecting IgG three- to four-fold better than to the control IgG purified from the serum of a blood donor, whereas peptides lacking the motif sequence showed between 1.0- and 1.5-fold higher binding with patient IgG (not shown). The relatedness to the gp41-epitope was proved by the competition shown by one of the "best" mimotopes with the epitope representative peptide No. 6357 (Fig. 3A, inset).

We used mimotopes No. 3 and No. 6 for further validation of their antigenic and immunogenic relatedness to the gp41 ID epitope. First, a panel of 22 IgG preparations from patients with disease progression was tested in an ELISA with mimotope No. 6 as antigen, of which 18 IgG recognized it reliably (Fig. 3B). This result is consistent with around 90% detection of HIV-1 infection by the synthetic peptide approach (Pan Chan Du et al., 2002) used in the routine diagnosis after being introduced by Gnann and coworkers (1987).

Second, the immunogenic relevance of mimotopes to the gp41 ID epitope was investigated by immunizing mice and assessing antisera for recognition of the epitope-representing peptide 6357. We immunized four groups of three mice: with

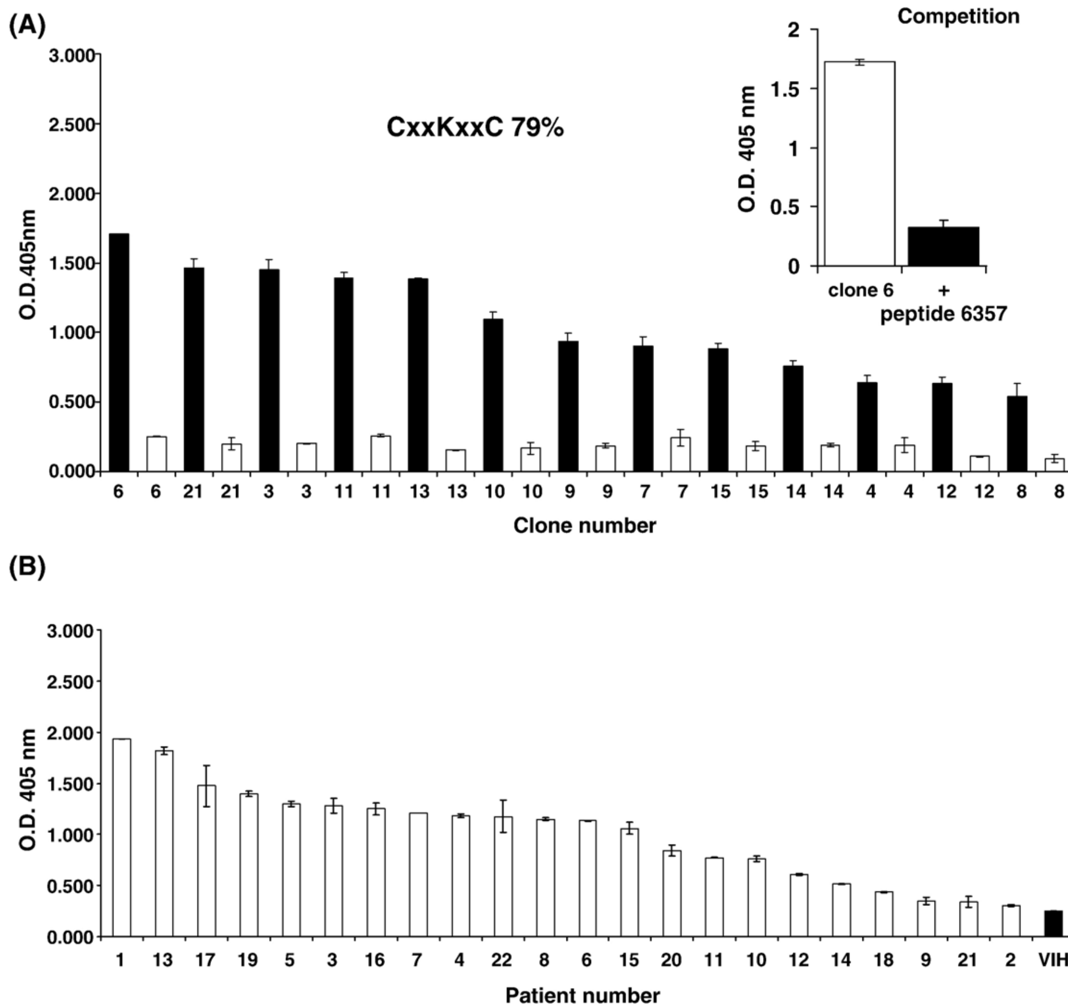

Fig. 3. Antigenic (A, B) and immunogenic (C) relatedness of mimotopes to the gp41 ID epitope. (A) Reactivity in ELISA of pre-HAART Pt 1 IgG (1–1) with the selected mimotopes. Mimotopes selected by IgG preparations 1–2 and 3–1 showed similar reactivity patterns with their IgG and are not shown. Filled bars – reactivity of IgG with mimotopes (see Fig. 2C for the sequences); open bars – reactivity of the same mimotopes with HIV-1(−) IgG. Inset: the effect of the peptide 6357 LLGFWGCSGKLICTT (5 µg/ml) on reactivity of Pt 1 IgG with mimotope 3 (LPQQACLGKLLC). (B) Mimotope No. 6 (FGCLGKLVCDPPY) used as antigen in ELISA for immunodetection of a panel of HIV-1(+) IgG. Open bars: IgG of 22 HIV(+) patients; filled bar: HIV-1(−) control. Error bars shown represent standard deviations from mean values shown by duplicate probes. (C) Assessment of the immunogenicity of mimotope collection. Bars consecutively show the reactivity with synthetic peptide 6357 (Fig. 1A) of pooled sera (three dilutions) from groups of three mice immunized with following mimotopes: No. 3 and No. 6 (Fig. 2C), portions of the eluates from selection with the pre-HAART IgG from Pt 1, and Pt 3, and reactivity of the peptide with pool of pre-immune sera of the mice.

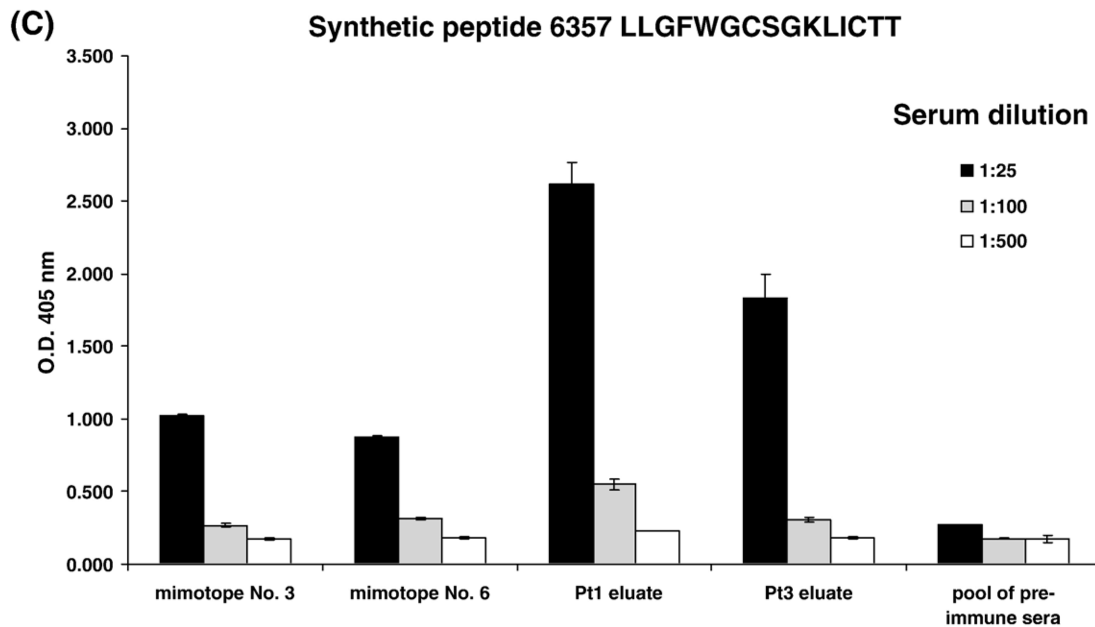

Fig. 3 (*continued*).

individual mimotopes (two groups) and with samples from whole eluate. ELISA data presented in Fig. 3C show the reactivity of pooled antisera of each of these groups in comparison with pooled pre-immune serum. Two individual mimotopes, No. 3 and No. 6, induced comparable reactivity to the test-peptide, whereas, unexpectedly, the eluates from selections using IgG from Pt 1 and Pt 3 induced two- to three-fold higher reactivity. Also notable is that the reactivity correlated with the proportion of CxxKxxC-bearing phages in the eluates (deduced from the % of CxxKxxC mimotopes in the sequenced samples, Table 1): the antisera of mice immunized with the Pt 1 eluate showed higher reactivity than the antisera raised against the eluate obtained with IgG from Pt 3. These properties of mimotopes strongly suggest that they induced in mice antibodies of similar specificity that possessed the antibody elicited by the gp41 ID epitope in patients, i.e. the mimotopes reproduced in the mouse system, the immunogenic specificity of the natural epitope.

Three findings are of interest in these experiments: (1) the higher epitope-related immunogenicity of the pool of the CxxKxxC peptides compared to individual peptides, (2) the recognition by the mimotope-induced mouse antibody of test-peptide (No. 6357, Fig. 1A) representing the gp41 epitope, (3) the positive correlation between the proportion of the CxxKxxC peptides in the Pt 1 (79%) and Pt 3 (26%) eluate samples (Table 1), and the reactivity to the epitope they induced in mice (Fig. 3C).

4. Discussion

The screening of phage-displayed random libraries with HIV-infected patient sera represents an important source of peptide mimics of HIV-1 immunodominant epitopes to extend the range of structural studies of viral epitopes and provide novel antigenic and immunogenic resources needed for the control of AIDS through immunodiagnosis, blockage of viral entry into target cell and induction of protective responses. These phage display prospects for HIV-1 have only recently begun to be explored (Scala et al., 1999; Enshell-Seijffers et al., 2001), and further progress will require efforts improving the efficiency, predictability and reproducibility of the selection of peptides by the patient's sera. In this study we applied phage display to a particular target, the immunodominant epitope of HIV-1, which plays an important part in the mechanism of viral infection, and served as an excellent model to demonstrate the successful application of the phage display to this virus. Results of the study add new information on the factors that may allow reliable and high-yield selection of mimotope collections for HIV.

These factors mostly concern the characteristics of the serum and the library. In our biopanning experiments with 9 IgG, only 3 were as successful as we desired, and these IgG preparations contained the highest levels of antibody. The implication from this limited success is that serum IgG may be as effective as monoclonals usually are in biopanning, but only when the titer of the selecting antibody to the epitope in IgG is above a threshold that in this case was determined empirically from many experiments; this represents the basis for obtaining future patient serum selectable peptide collections for this important epitope. Furthermore, we found that the selection efficiency was proportional to the antibody titer, not only making it practical to eliminate negative experiments but also to predict from this titer the yield of mimotopes. HAART strongly suppressed this ability and, generally, sera of patients undergoing HAART are not potent, or only potent during the first few weeks of the therapy.

The library used also affected the results of biopanning. No phage sequences containing the CxxKxxC motif were isolated using 7 mer libraries, either linear or constrained, despite the expectation that the cyclic peptides presented by the 7 mer

constrained library would provide good possibilities for selection of cyclic peptides similar to the gp41 ID epitope. Our explanation for this is that the ring size is one of the most critical requirements; the importance of cyclization for affinity has been previously noted (Pan Chan Du et al., 2002; Menendez et al., 2004). However, in this particular case, the gp41 ID epitope, libraries of cyclic peptides with a ring-size larger than the epitope, even containing only one additional residue, were not suitable in the biopanning of the 7 mer constrained library in our study and of a 12 mer constrained library screened by Enshell-Seijffers et al. (2001).

Based on published data concerning the HIV-1 gp41 epitope (Gallagher et al., 1989; Oldstone et al., 1991; Stigler et al., 1995; Pan Chan Du et al., 2002), one can see that the mimotopes described here reflect well the primary and conformational structural features of the HIV-1 gp41 epitope. One of the most critical features are the two cysteines separated by five residues of which the central lysine is shown by many data to be most critical. In some instances antibodies to the epitope could recognize sequences lacking one of the cysteines (Scala et al., 1999). Also, as reported by Oldstone and coworkers (1991), serum of 8% of HIV-1-infected patients recognized along with the lysine-containing, type 1, CSGKLIC peptide also type 2 CAF**R**QVC sequence with arginine replacing the lysine. Consistent with this observation, in the experiments of Stigler et al. (1995) with the 3D6 Fab (recognizes the gp41 ID epitope) a CSG**R**HIC-containing peptide showed the same K_d (1×10^{-6} mol/L) level as the peptide containing CSG**K**HIC. In a comprehensive study done by the latter group each p41 CSGKLIC epitope residue was substituted by all L- or D-amino acids and it was found that lysine in the peptide could be replaced by other amino acids including alanine. More recently, Pan Chan Du et al. (2002) have shown HIV-1(+) patient serum to recognize certain b-analogues of the gp41 ID epitope better than the natural epitope peptide. These results demonstrated that, although the gp41 ID epitope is conserved, it elicits antibody able to cross-react with various epitope analogues including peptides lacking lysine or one of the cysteines. The majority of the data obtained in these studies, however, showed that lysine and two cysteines were needed for specific interaction with antibody. Two cysteines were needed to ensure formation of a loop with specific conformation rendered by characteristic orientation of the Lys, Ile, and Leu side chains (Pan Chan Du et al., 2002). According to this hypothesis, the unique orientation of the side chain of the central lysine represents the key structural element of the natural epitope that its mimics should present. Detailed spatial structure comparison of the epitope and its analogues complexed with epitope-specific Ab would be of considerable interest to reinforce these observations. In the absence of such structural data, successful selection from a random library of peptides provides information on structural elements most important for the binding. When in our biopanning experiments serum screened a 12 mer random library containing many different gp41 epitope analogues (including those lacking the central lysine or one of the cysteines) it preferentially selected double-cysteine epitope mimics with lysine at central position. The preference towards these sequences might additionally be realized due to exposure of the CxxKxxC loops on the tip of gp3, via the flexible GGGS linkers permitting accommodation of the peptide to the antibody paratope. Through the molecular modeling of mimotopes (underway in our laboratory), we expect to have more detailed knowledge about the specific tertiary structure of the loop, which supposedly includes peculiar side chain positioning (Pan Chan Du et al., 2002) and a beta-turn (Oldstone et al., 1991) as determinants of its antigen specificity.

In this study mimotopes were selected for the gp41 ID epitope for three reasons.

1. To investigate the gp41–gp120 interaction. It has been suggested that within the position in the HIV-1 envelope to which the gp41–gp120 complex locates, the gp41 loop-epitope is inserted into a “pocket” presumed to exist in the gp120 C5 domain (Helseth et al., 1991; Schulz et al., 1992). This hypothesis is supported by recent experiments, in which cysteines introduced into the two proteins formed bridges between gp120 and gp41 predominantly around the gp41 loop region (Binley et al., 2000). Experiments can be designed using mimotopes, such as engineered loop versions, to model this contact and to verify how different amino acids that contain mimotopes influence the tightness of the interaction. The importance of this application lies in the novel opportunity of investigating the mechanism of the conformational transition from gp120 to gp41 that is triggered by the interaction of the envelope complex with target cell and which ends in membrane fusion.
2. Novel diagnostic antigens. Although serodiagnosis by means of synthetic peptides carrying the CSGKLIC sequence is widely practiced, experiments are underway to test the ability of our mimotopes displayed on phage to complement or perhaps substitute for the synthetic peptides, which are known to suffer from certain drawbacks (see Introduction). Our first data with a panel of 22 patient sera (Fig. 3B) showed that one of the mimotopes used as antigen was recognized by about 82% of patients, which is comparable with the detection with synthetic peptides (Gnann et al., 1987; Pan Chan Du et al., 2002). Our hypothesis that a collection of mimotopes can provide better antigenicity for diagnosis, relies on the evidence that amino acid substitutions in CSGKLIC sequence may result both in decreased and increased antigenicity (Pan Chan Du et al., 2002), and also on the fact that the mimotope collection contains a lot of substitutions to explore, which gives the possibility to enhance their diagnostic value. The microbiological technology exploiting the phage reproduction potential would be in general more economical and easier to manage than the chemical synthesis of peptides to supply diagnostic antigens.
3. Novel immunogens. The controversy between the outstanding immunodominance of the gp41 loop-epitope (known since 1989, Gallagher et al., 1989) and the experimental evidence that the antibody it elicits does not neutralize the virus (Burrer et al., 2005), contributes to the epitope’s continued obscurity. Burrer et al. (2005) demonstrated that this epitope is the major region in the virus that binds serum antibody. In our experiments with mice, the mimotopes effectively induced new antibodies of the same specificity that were present in patient serum (Fig. 3C). Hence, they can

be used as engineered immunogenic versions of the epitope to investigate, in laboratory animals, whether amino acid substitutions in the epitope can modify the properties of the induced antibody towards the neutralization. Our finding that when the whole contingent of selected library peptides was injected into mice the immunogenicity was higher than with individual mimotopes (Fig. 3C), shows that the immunogenicity of mimotopes are different and additive when they work collectively; this opens a possibility of immunizing animals with different mimotope combinations and searching for inducers of neutralizing antibodies in adequate assay systems.

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