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An Endogenous HIV Envelope-derived Peptide without the Terminal NH_3^+ Group Anchor Is Physiologically Presented by Major Histocompatibility Complex Class I Molecules*

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Cytotoxic T lymphocytes (CTL) recognize viral peptidic antigens presented by major histocompatibility complex (MHC) class I molecules on the surface of infected cells. The CTL response is critical in clearance and prevention of HIV infection. Yet, there are no descriptions of physiological peptides derived from the viral envelope protein. In the few reports on endogenous MHC class I viral peptidic ligands from HIV internal proteins, definitive positive identification by mass spectrometry is lacking. The HIV-1 envelope glycoprotein gp160 induces a strong specific CTL response restricted by several human and murine MHC class I molecules, including H-2D^d. Previous analyses showed that this response can be optimally mimicked with the synthetic decameric peptide ^{318}RGPGRAFVTI^{327}. We aim to identify the endogenous natural peptides mediating the response to this epitope. Our data indicate the presence of, at least, two peptidic species of different length and sharing the same antigenic core, which are associated with the D^d presenting molecule in infected cells. One species is at least, probably, the optimal decapeptide. The second species, identified by mass spectrometry for the first time in HIV, is, unexpectedly, a nonamer, which lacks the correctly positioned N-terminal group to bind to D^d. And yet, it is present in similar amounts and, notably, is equally antigenic. Thus, the physiological set of HIV-derived MHC class I ligands is richer and different than expected from studies with synthetic peptides. This may help raise the plasticity and thus the effectiveness of the immune response against the viral infection. These data have implications for HIV vaccine development.

The cellular immune response mediated by $CD8^+$ cytotoxic T lymphocytes (CTL)[1] plays an important role in controlling HIV-1 infection (1, 2). There is a temporal correlation between vigorous CTL responses and initial control of HIV replication during primary infection (3, 4). Also, there is an important presence of CTL response in long-term nonprogressors compared with progressors (5, 6). In addition, in SIV-infected macaques the abrogation of the $CD8^+$ population induces a high increase in viral load that in turn causes animal death (7). Furthermore, homozygosity at any HLA locus is strongly associated with rapid progression to disease (8). In addition, some loci such as HLA-B35 (8) and -B27 (9) are individually associated with faster and slower development of AIDS, respectively. The virus escapes this CTL response (10, 11), which confirms that $CD8^+$ T lymphocytes exert an immunological pressure (12). This evidence indicates a protective role of CTL in preventing and controlling HIV infection. For this reason, characterization of epitopes recognized by CTL represents an important contribution in the development of therapeutic agents such as vaccines against the virus. Identification of the naturally processed epitopes will assure their correct inclusion in vaccine formulations, and help improve the efficiency of endogenous antigen presentation by MHC class I molecules to potentiate the CTL response. Notably, there are no reports of identification of endogenous peptides derived from any epitope recognized by CTL in the HIV envelope protein, and very few descriptions from HIV internal proteins (13–15).

In the BALB/c mouse model, the HIV-1 strain IIIB envelope glycoprotein gp160 induces a CTL response restricted by the H-2D^d class I molecule (16). Analysis of overlapping synthetic peptides covering practically all the gp160 sequence revealed that the 15mer peptide ^{315}RIQRGPGRAFVTIGK^{329} (then named p18) was the major one recognized by CTL (16). Afterward, additional experiments described the short ^{318}RGPGRAFVTI^{327} synthetic peptide (herein named R10I) as the most antigenic within this sequence (17–21). This region is particularly interesting because it contains epitopes for at least 13 different MHC class I molecules from various species (6 murine, 5 human, 1 chimpanzee, and 1 macaque), including HLA alleles frequent in the human population (HLA-A2, -A3, -A24, -A11, and -A30) (22). Thus, this viral region, known as V3, is recognized by CTL from an important fraction of patients, which is a paradox because it is one of the regions of the HIV genome that is more variable among virus strains.

However, all experiments have been performed with synthetic peptides. We are interested in the study of the natural peptides responsible for this D^d-restricted, envelope glycoprotein-specific, CTL response. This report shows that the endogenous proteolytic processing of gp160 protein for presentation to CTL generates at least two viral-derived peptidic species bound to the presenting molecule in infected cells. One of them may correspond to the optimal synthetic peptide, while a second one is an unexpected shorter species. In addition, our

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[1] The abbreviations used are: CTL, cytotoxic T lymphocytes; RP-HPLC, reversed-phase high-performance liquid chromatography; WR, Western Reserve; BFA, brefeldin A; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; m.o.i., multiplicity of infection; rVV, recombinant vaccinia virus; PFU, plaque-forming unit; MHC, major histocompatibility complex; HIV, human immunodeficiency virus; ICS, intracellular cytokine staining.

results exclude as natural D^d ligand at least one synthetic peptide that is as antigenic as the natural peptides. These results underscore the need to study the peptides resulting from physiological processing, because the natural situation can be more complex than the optimum detected with synthetic peptides.

EXPERIMENTAL PROCEDURES

Mice—BALB/c mice (H-2^d haplotype) were bred in our colony.

Cell Lines—All cell lines were cultured in IMDM supplemented with 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol. The P13.1 cell line is a derivative from mouse mastocytoma P815 cells (H-2^d) by transfection with the *lacZ* gene encoding β-galactosidase (23). For infection, untransfected murine kidney L cells (Ltk^- cells) and L cells transfected with D^d were used (24). For stability assays, the TAP-deficient human lymphoblastoid T2 cells were employed (25).

Synthetic Peptides—Peptides were synthesized in a peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA), and purified, when needed, by cation-exchange and/or reversed-phase HPLC (RP-HPLC). They were quantitated by A_{280} absorbance using peptide R10I as a standard, and the identity confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. All peptide sequences were derived from the sequence ^{313}KIRIQ**RGPGRAFVTI**GKIGNMRQAH^{337}, which forms part of the V3-loop antigenic area from HIV-1 strain IIIB envelope glycoprotein. They are named indicating the first amino acid, the length, and the last amino acid. Thus, R10I refers to the decamer of sequence ^{318}RGPGRAFVTI^{327}. See Table I for an overview.

Recombinant Vaccinia Viruses (rVV)—rVV-ENV (vSC25) encodes ENV envelope glycoprotein gp160 from the strain IIIB of HIV-1 (27). Its DNA sequence was confirmed. rVV-eC-10env encodes the HIV-1 strain IIIB ENV 318–327 epitope, corresponding to the decamer sequence R10I, inserted into the carboxyl end of HBe. The sequence at that site is ...RRSQS**RGPGRAFVTI**RESQC (28). The negative control rVV-HBe encodes the carrier protein HBe, the hepatitis B virus secretory core protein (29). rVV-encoded proteins relevant to this study contain their respective signal sequences for translocation into the endoplasmic reticulum. All foreign genes cloned into the rVV used in this study are under the control of the vaccinia early-late promoter 7.5k. rVV-HBe and rVV-eC-10env are based on vaccinia Copenhagen strain, whereas rVV-ENV and rVV-D^d (30) present vaccinia Western Reserve (WR) background.

T Cell Lines and Cytotoxicity Assays—Polyclonal ENV-IIIB-monospecific CTL were generated by immunization of mice with 5×10^7 plaque-forming units (PFU) of rVV-ENV, followed by weekly restimulation of splenocytes with 10^{-6} M G9I synthetic peptide and interleukin 2, as described elsewhere (28). They were used as effector cells in standard 6-h cytotoxicity assays. Identical cytolytic pattern with different IIIB ENV 317–327 epitope peptides was found by restimulation with R10I (data not shown). As targets, L/D^d cells were infected overnight with rVV as described (31). For peptide titrations, P13.1 targets and peptides were incubated for 20 min and CTL were then added. When brefeldin A (BFA) (Sigma-Aldrich) was employed to block transit of MHC/peptide complexes along the secretory pathway, the percentage of specific inhibition was calculated as shown in Equation 1,

$$\% \text{ Specific inhibition} = \frac{[E - N] - [Eb - Nb]}{[E - N]} \times 100 \qquad \text{(Eq. 1)}$$

where E is lysis rVV^{ENV}, N is lysis $rVV^{NEGATIVE\ CONTROL}$, Eb is lysis rVV^{ENV} + BFA, and Nb is lysis $rVV^{NEGATIVE\ CONTROL}$ + BFA.

BFA was used as described (28), except that it was added in a 5-fold higher concentration during the preincubation time (30 min before addition of the virus) than throughout infection.

Isolation of Naturally Processed Peptides—L cells (3×10^9 in 30 roller bottles) were infected with rVV at a multiplicity of infection (m.o.i.) of 3.5 PFU per cell, and 16 h later naturally processed peptides were extracted from whole cells with trifluoroacetic acid (29, 32) and purified by RP-HPLC (33). Control extractions were performed identically, except that pellets of 10^9 P13.1 cells received 10 nmol of R10I and Q11I peptides immediately after addition of trifluoroacetic acid. Pooled RP-HPLC fractions that tested positive in cytotoxicity assays with P13.1 target cells and ENV-specific CTL were newly chromatographed by cation-exchange chromatography in a MonoS PC1.6/5 column (Amersham Biosciences). Elution was accomplished with a NaCl gradient in 7 mM sodium acetate, pH 4, 30% acetonitrile. Fractions from this second column and dilutions thereof were analyzed in new cytotoxicity assays. As internal standard, an ENV-unrelated peptide was included in all HPLC runs. To ensure comparability of different runs, the actual conductivity gradient was always monitored. Contamination of the HPLC columns was excluded by testing preceding HPLC runs with CTL. For standardization, serial dilutions of synthetic peptides were tested always in parallel.

Mass Spectrometry—HPLC fractions containing peptides from the cellular extracts were dried and dissolved in 5 μl of 0.1% formic acid in methanol:water 1:1 and sequenced by Dr. J. Vázquez (Centro de Biología Molecular, Madrid, Spain) by quadrupole ion trap nanoelectrospray MS/MS in an LCQ mass spectrometer (Finnigan ThermoQuest, San José, CA). The charge and the mass of the ionic species were determined by high-resolution sampling of the mass/charge rank. Collision energy and ion-precursor resolution were improved to optimize the fragmentation spectrum.

MALDI-TOF mass spectrometry was performed in a Reflex instrument (Brucker-Franzen Analytik, Bremen, Germany) operating in the positive ion reflection mode, by Dr. J. P. Albar (Centro Nacional de Biotecnología, Madrid, Spain). Twenty-five microliters of a given HPLC fraction were dried, resuspended in 2 μl of 0.1% trifluoroacetic acid in a 2:1 solution of water:acetonitrile, and mixed with 2 μl of saturated α-cyanohydroxycinnamic acid matrix in the same solution. One microliter of the mixture was dried and subjected to analysis.

MHC/Peptide Stability Assays—TAP-deficient T2 cells were infected at 26 °C with rVV-D^d at a m.o.i. of 3, and 14 h later they were washed and incubated for 2 h at 37 °C in phosphate-buffered saline containing 0,1% bovine serum albumin and 500 μM of the different peptides, as described (32). After washing (time point 0), the cells were further incubated at 37 °C. Aliquots removed at different time points were stained with mAb 34-5-8S, which recognizes D^d bound to peptides (34), followed by flow cytometry (32). Cells incubated without peptide had peak fluorescence intensities close to background staining with second Ab alone. Fluorescence index was calculated at each time point as the ratio of peak channel fluorescence of the sample to that of the control incubated without peptide.

Intracellular Cytokine Staining (ICS)—Intracellular cytokine staining assays were performed as described previously (35). Splenocytes were obtained from BALB/c mice 7 or 33 days after infection with 5×10^7 PFU of rVV, stimulated with 10^{-5} M peptide for 30 min or for 2 h, respectively, and stimulated for a further 3 h in the presence of 10 μg/ml BFA. The assays were performed in the absence of fetal calf serum. Following stimulation, cells were incubated with FITC-conjugated anti-$CD8^+$ (clone 53–6.7) mAb for 30 min at 4 °C, fixed with Intrastain kit (DakoCytomation, Glostrup, Denmark) reagent A, and incubated with PE-conjugated anti-IFN-γ mAb in the presence of Intrastain kit permeabilizing reagent B for 30 min at 4 °C. All antibodies were purchased from BD PharMingen (San Diego, CA). Events were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using CellQuest software (BD Biosciences).

RESULTS

Presentation of the HIV-1 Envelope Glycoprotein Requires Endogenous Processing—In BALB/c mice the CTL response against HIV-1 strain IIIB envelope glycoprotein is restricted by the D^d class I molecule (16). Glycoprotein-specific CTL recognize ^{318}RGPGRAFVTI^{327} decamer-pulsed target cells (17, 18), and other extended nested peptides from the same antigenic region (18, 20). Infection of L/D^d cells with a rVV that codes for the full-length glycoprotein, rVV-ENV, induces specific recognition by CTL (Fig. 1*A*, *squares*). In addition, the decamer ^{318}RGPGRAFVTI^{327} (named R10I thereafter) is efficiently presented both as exogenous synthetic peptide (66% specific lysis *versus* 0% without peptide at an E:T ratio of 5:1) and also when included in the C terminus of the heterologous HBe secretory protein and expressed endogenously by rVV-eC-10env (Fig. 1*A*, *circles*).

In order to confirm that the presentation of the envelope glycoprotein in L/D^d cells requires endogenous processing, cytotoxicity assays were performed in the presence of BFA. This drug blocks class I export beyond the *cis*-Golgi compartment (36), thus preventing the expression of newly endogenously assembled MHC/peptide complexes at the membrane. Exogenously added peptides are not affected by BFA, and thus no

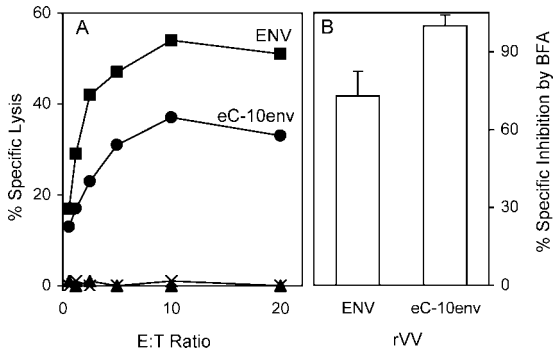

FIG. 1. **Endogenous presentation of ENV by the MHC class I molecule D^d.** *Panel A*, recognition of ENV by CTL. L/D^d cells were infected overnight with rVV-ENV (■) or with rVV-eC-10env, which encodes the R10I epitope sequence (●), at a m.o.i. of 10, and were analyzed with ENV-specific CTL in a cytotoxicity assay. rVV-HBe (▲) and WR (x) were employed as negative controls at the same m.o.i. As a positive control, recognition by CTL of WR-infected cells prepulsed with 10^{-5} M R10I synthetic peptide gave lysis values of 65, 71, and 66% at E:T ratios of 20:1, 10:1, and 5:1, respectively. *Panel B*, inhibition by BFA (5 μg/ml) of the recognition of the ENV epitope by CTL in rVV-infected cells. The data are mean values of at least two experiments.

inhibition was found when WR-infected cells pulsed with R10I peptide were treated with BFA (data not shown). In contrast, Fig. 1*B* shows a significant decrease in the recognition of the native glycoprotein (73 ± 9.5%) and the chimeric construct (100 ± 4.2%) when infection with the respective rVV was performed in the presence of BFA. Thus, CTL recognition of this ENV epitope in association with the D^d molecule requires processing of endogenously synthesized proteins in infected L/D^d cells, similarly to other cell lines (28, 37).

The Endogenously Processed Peptides Are Associated with D^d in the Infected Cell—In order to identify the natural endogenously processed peptides of the gp160 protein that are associated with the D^d molecule, an acid extraction of the peptides generated after infection of L/D^d cells with rVV-ENV was performed. The peptides were separated by RP-HPLC (Fig. 2) and analyzed in cytotoxicity assays. This analysis showed a major antigenic activity around fraction 16 (Fig. 2, *closed symbols*). The CTL did not recognize WR-infected target cells, indicating the specificity of the antigenic peak.

Whole cells rather than antibody-selected D^d were used for natural peptide extraction, in order not to exclude potential precursor peptides. Because of this, it was interesting to test whether this antigenic activity was detected only when D^d was present. Therefore, extraction experiments were carried out using the D^d-negative parental Ltk^- cells, using similar infected cell equivalents as tested previously with D^d-positive cells, and CTL of confirmed identical sensitivity. No antigenic peaks were found in the RP-HPLC runs from infected Ltk^- cells (Fig. 2, *crosses*). These results indicate that peptides generated by endogenous processing of the envelope glycoprotein in infected L/D^d cells and detected in Fig. 2 must be bound to the presenting D^d molecule, and are therefore neither free from MHC, nor bound also to the endogenous $H\text{-}2^k$ molecules of L cells.

Physiological Processing Generates at Least Two Different D^d-associated Peptidic Species, One of Them Unexpectedly Monocharged—Even though the gradient used for elution of the peptides from the RP-HPLC column was rather flat, several different overlapping synthetic peptides from the antigenic region encompassing ENV residues 313–337 eluted near fraction 16 (see below). This is probably because all these peptides have related, low hydrophobicity, as all of them are positively charged. This impeded further characterization of the endogenous peptides generated. Therefore, in an attempt to resolve

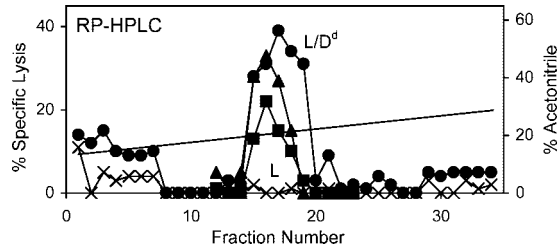

FIG. 2. **Natural endogenous peptides generated after processing of ENV in D^d-positive cells.** Peptides extracted from L/D^d cells infected with rVV-ENV at a m.o.i. of 3.5 were separated by RP-HPLC. Fractions were tested with ENV-specific CTL to analyze the presence of antigenic peptides. The results obtained with 2.88×10^8 (●), 1.44×10^8 (▲), and 0.72×10^8 (■) cell equivalents per well of the cytotoxicity assay are depicted. An identical experiment was carried out with 1.16×10^8 infected cell equivalents of the D^d-negative Ltk^- cells (x, L). At right, the secondary scale indicates the monitored acetonitrile gradient (*solid line*) employed for elution.

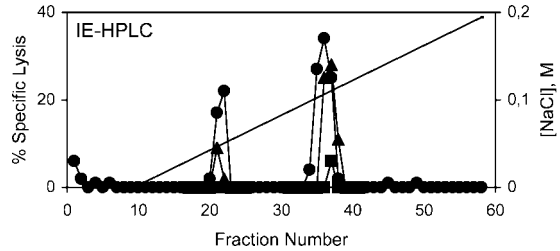

FIG. 3. **Different peptidic species result from endogenous ENV antigen processing.** Antigenic fractions 14–22 from the RP-HPLC run shown in Fig. 2 were pooled and separated by cation-exchange chromatography (IE-HPLC). This was followed by a cytotoxicity assay of the collected fractions. The results obtained with 1.13×10^8 (●), 0.56×10^8 (▲), and 0.28×10^8 (■) cell equivalents of the peptide extracts from D^d-positive cells infected with rVV-ENV virus are plotted. *Right*, the secondary scale indicates the monitored conductivity of the NaCl gradient (*solid line*) employed for elution.

the peptidic species that correspond to the antigenic peak that was obtained in the RP-HPLC column, the pool of fractions 14–22 containing antigenicity as revealed by the cytotoxicity assay (Fig. 2) was newly chromatographed by cation-exchange HPLC. Fig. 3 shows two different antigenic peaks generated as a result of the endogenous processing of the envelope glycoprotein in L/D^d cells. The first antigenic activity (fractions 21–22) corresponds to monocharged species, while the second peak (fractions 35–37) groups some bi-charged and some tri-charged peptides. Synthetic peptides from this antigenic area, up to 15 residues long, were tested to also set the elution positions for other peptidic species with up to 4 positive charges. Accordingly, further fractions were collected up to number 60 to cover peptidic species up to 4 charges, but these contained no antigenicity (Fig. 3).

The first antigenic activity (fractions 21–22) in the ion exchange column coelutes with the only two monocharged synthetic peptides from this area that contain the epitope core: G10G and G9I (see Table I for a summary of results). Shorter mono-charged peptides P8I and G7I also co-elute (data not shown). Since G10G elutes away from the antigenic fractions in RP-HPLC, we excluded this peptide from further analysis. We next tested the recognition of the three peptides that coelute with the antigenic peaks in both HPLC analyses. Fig. 4*A* shows that G9I is efficiently recognized by specific CTL, with a similar titration curve as R10I (see below, Fig. 7). In contrast, P8I was recognized 1000-fold less than G9I, and G7I was not recognized at all, as also found by Bergmann *et al.* (19). G10G, already excluded as a candidate natural peptide, was essentially not recognized either (Table I). Because reports in the literature (18–20) sharply disagree on the antigenicity of G9I, it was

TABLE I
Functional properties of synthetic peptides and overview of results

Synthetic peptide[a]		Termini[b]		Coelution with antigenicity in RP-HPLC	Antigenic peak of coelution in IE-HPLC[c]	Positive charges[d]	CTL recognition[e]	D^d/peptide stability[g]
Name	Sequence	N-end	C-end					
R10I	**RGPGRAFVTI**	–	–	**Yes**	**2nd**	2*[d]	++++[e]	+++[g]
R11G	RGPGRAFVTIG	–	Extended	No	2nd	2*	+++	n.d.[h]
R12K	RGPGRAFVTIGK	–	Extended	No	None	3*	+++[f]	n.d.
Q11I	**QRGPGRAFVTI**	Extended	–	**Yes**	**2nd**	2*	++++	+++
I12I	IQRGPGRAFVTI	Extended	–	Yes	None	2	++++	+++
R13I	RIQRGPGRAFVTI	Extended	–	Yes	None	3*	+++	n.d.
G9I	**GPGRAFVTI**	Deleted	–	**Yes**	**1st**	1	++++	++
G10G	GPGRAFVTIG	Deleted	Extended	No	1st	1	+	n.d.
G11K	GPGRAFVTIGK	Deleted	Extended	No	None	2	++[f]	n.d.
G12I	GPGRAFVTIGKI	Deleted	Extended	Yes	None	2	+	n.d.
Q12G	QRGPGRAFVTIG	Extended	Extended	No	2nd	2*	+	n.d.
I14K	IQRGPGRAFVTIGK	Extended	Extended	No	2nd	3	++[f]	n.d.

[a] Not all tested synthetic peptides are included. Nine peptides that do not add information are not summarized here, notably those with longer extensions.
[b] The terms deleted or extended are in reference to the termini of the boldface R10I peptide (first peptide in the table).
[c] Two antigenic peaks were detected in cation-exchange HPLC (IE-HPLC) runs of infected cell extracts, as shown in Fig. 3, and are referred here to as the 1st and 2nd peak. When synthetic peptides were individually analyzed by cation-exchange HPLC, its elution in any of the fractions of either peak is scored here as coelution. Peptides with 1 positive charge coelute with the 1st peak. Peptides with 2* (see footnote *d*, positive charges coelute with the 2nd peak (Fig. 6).
[d] The numbers refer to the number of positively charged residues (Arg, Lys, or His) in the peptide. An asterisk indicates that the positive residue is in position 1 or 2 of the peptide, since this property markedly retards elution in cation-exchange HPLC.
[e] Peptides were serially diluted in 10-fold steps, and sequentially incubated with targets and CTL. Each additional + sign indicates a 10-fold higher antigenicity of the respective peptide.
[f] A peptidyl-carboxydipeptidase activity present in fetal calf serum removes the -GK *C*-terminal extension (26), and artificially improves antigenicity of several peptides tested in this series.
[g] MHC/peptide stability assays were performed as indicated in "Experimental Procedures." The + signs have a qualitative meaning: +++: abundant and stable complexes; ++: abundant but slightly less stable complexes. See Fig. 7*B* for the actual comparative data.
[h] n.d., not done.

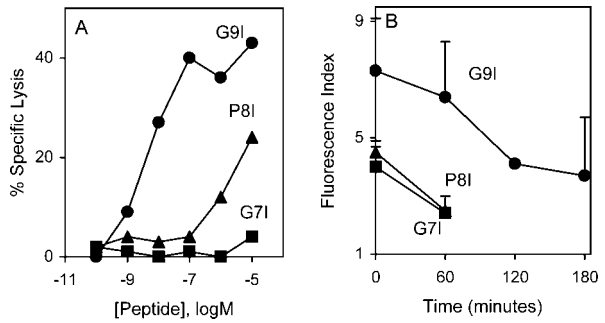

FIG. 4. **Exogenous presentation and MHC stability assay of mono-charged synthetic ENV peptides.** *Panel A*, Recognition by CTL of titration curves of G7I (■), P8I (▲), and G9I (●) synthetic ENV peptides. *Panel B*, stability of cell surface MHC/peptide complexes measured by flow cytometry. Peptides: same code as in *panel A*. Results are the mean of at least three experiments.

tested and excluded that our G9I was contaminated with a more antigenic species, specifically with the bi-charged R10I peptide, by using both mass spectrometry and CTL testing of cation-exchange HPLC runs of either G9I or R10I peptides separately (data not shown).

In addition, MHC/peptide complex stability assays using TAP-deficient T2 cells infected with rVV-D^d were carried out. Fig. 4*B* shows that G9I induces a high number of complexes with the presenting molecule whereas both P8I and G7I induce much fewer and markedly less stable complexes. Therefore, the short P8I and G7I peptides cannot be considered reasonable candidates for natural peptides and, thus, the first antigenic activity detected by ion exchange HPLC must be assigned to the G9I peptide.

This preliminary conclusion was validated by mass spectrometry analysis of the cation exchange column antigenic fraction. Despite the exposure of the cellular extracts to NaCl during peptide elution (Fig. 3), which interferes with ionization, and despite the presence of an R in the peptide sequence, which disfavors detection of the N-terminal fragments, quadrupole/ion trap mass spectrometry showed that fraction 21 unequivocally contained G9I peptide (Fig. 5). In contrast, the octamer P8I was not detected. These experiments endorse the antigenicity and complex formation assays (Fig. 4 and Table I) as a valid approach to the identification of naturally processed peptides. In conclusion, this result definitively confirmed that the G9I nonamer was endogenously processed from HIV ENV glycoprotein and associated to D^d in infected cells.

Some authors observed the strong antigenicity of the G9I peptide, almost comparable to that of the R10I decamer (18, 19). However, another group reported later that this monocharged nonapeptide was essentially not antigenic (20), and this latter report was widely accepted as an indication that the optimal synthetic peptide was, by far, R10I. This was supported by the excellent fit of the R10I peptide into the D^d molecule, as revealed by crystallographic studies (38, 39). On this light, it was unexpected to find this monocharged G9I species as a result of endogenous processing. The discordance in the reports (18, 19) (and this report), *versus* (20) might be due to differences in the experimental settings. Our findings now of the G9I synthetic peptide as strongly antigenic and capable of inducing MHC/peptide complexes and, most importantly, our identification by mass spectrometry of G9I as one of the endogenously processed peptides, finally settle this controversy. In addition, these results underlie the importance of identifying the naturally processed peptides because results with synthetic peptides may bias further research.

The Second Set of Bi-charged, D^d-associated Peptidic Species—The second antigenic peak of the cation-exchange column (fractions 35–37, Fig. 3) coelutes with those bi-charged species that have a positive charge directly at the N-terminal residue or in position 2. Close proximity of the positive charges in the peptide promotes repeated binding of the peptide molecules to the anionic residues in the HPLC column resin, additionally retarding their elution. Most bi-charged synthetic peptides did not coelute with this natural peptide peak, but at earlier fractions, around fraction 28, as their positively charged residues

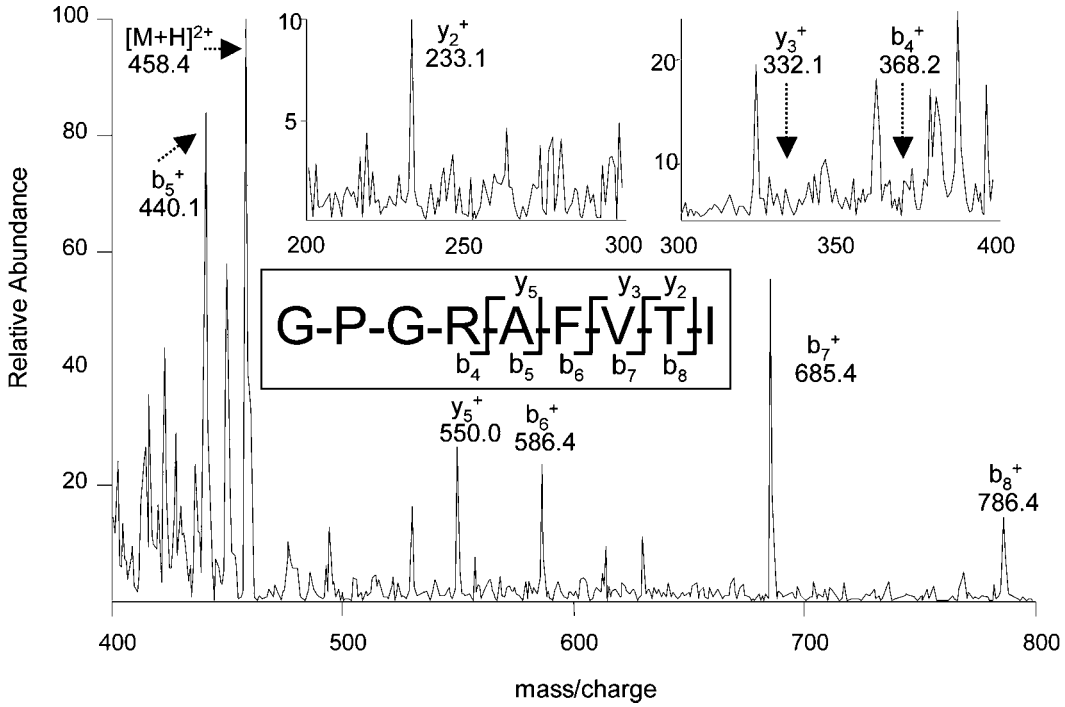

FIG. 5. **Identification by mass spectrometry of G9I in infected cell extracts.** MS/MS fragmentation spectrum obtained after quadropole ion trap mass spectrometry of fraction 21 of the cation-exchange run of extracts of L/D^d cells infected with rVV-ENV and shown in Fig. 3. The *vertical axis* represents the relative abundance of each fragmentation ion with respect to the parental ion corresponding to the G9I peptide, which has a mass/charge ratio of 458.4. Ions generated in the fragmentation are detailed for different mass/charge intervals in the *three panels*, while the sequence deduced from the indicated fragments is shown in the *central box*.

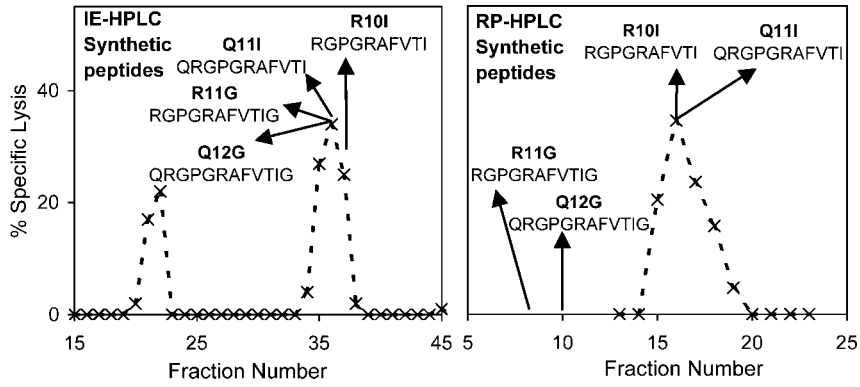

FIG. 6. **Elution of bi-charged ENV synthetic peptides in HPLC gradients.** Different bi-charged ENV synthetic peptides, as indicated, were analyzed independently by cation-exchange HPLC (IE-HPLC, *left panel*) or RP-HPLC (*right*). *Arrows* indicate fraction of elution of the A_{280} peak. For reference, CTL results (x) with natural, extracted peptides from 1.13×10^8 (*left*) or 0.72×10^8 (*right*) cell equivalents of L/D^d cells infected with rVV-ENV at a m.o.i. of 10 are shown.

are located further away from position 1. As for monocharged peptides, similar analyses of bi-charged synthetic peptide coelution in both ion exchange-HPLC and RP-HPLC systems were performed (Fig. 6, *A* and *B*, respectively). From 11 possible bi-charged ENV peptides containing the antigenic core, one was excluded because of its deduced irrelevant elution profile, and 10 were tested. From these, only four had a positive charge at position 1 or 2, and only these four peptides coeluted with the natural antigenic activity from the ion exchange column (Fig. 6*A*). Only two of these, R10I and Q11I, which differ between them by one N-terminal residue, are compatible with the RP-HPLC antigenic peak (Fig. 6*B*). Unfortunately, attempts to discriminate between R10I and Q11I peptides by analyzing the antigenic fractions by mass spectrometry were compromised by the lack of sensitivity. Indeed, fragmentation of synthetic R10I and Q11I is at least 10-fold less efficient than that of synthetic G9I (data not shown). The presence of an additional R in these two peptides as compared with G9I may have contributed to further decreased efficiency of detection of fragmented ions, already close to detection limit for the natural G9I peptide. However, the detection of a signal corresponding to the fragmentation ion of the C-terminal tetrapeptide FVTI of the R10I peptide was compatible with the presence of this decameric molecular species in fraction 37 of the cation-exchange run.

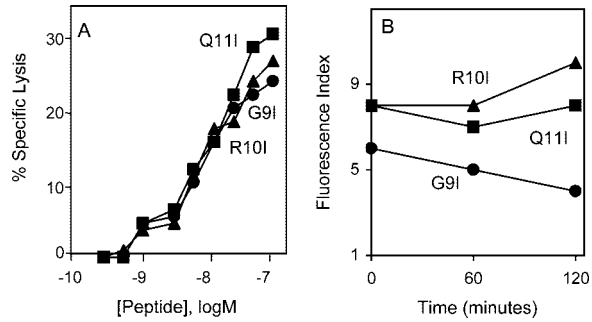

FIG. 7. **Exogenous presentation and MHC stability assay of bi-charged synthetic ENV peptides.** *Panel A*, recognition by CTL of titration curves of bi-charged R10I (▲), and Q11I (■) synthetic ENV peptides compared with the mono-charged G9I (●) peptide. *Panel B*, stability of cell surface MHC/peptide complexes assessed by flow cytometry. Peptides: same code as in *panel A*.

In addition to some bi-charged peptides (Table I, 2* charges),

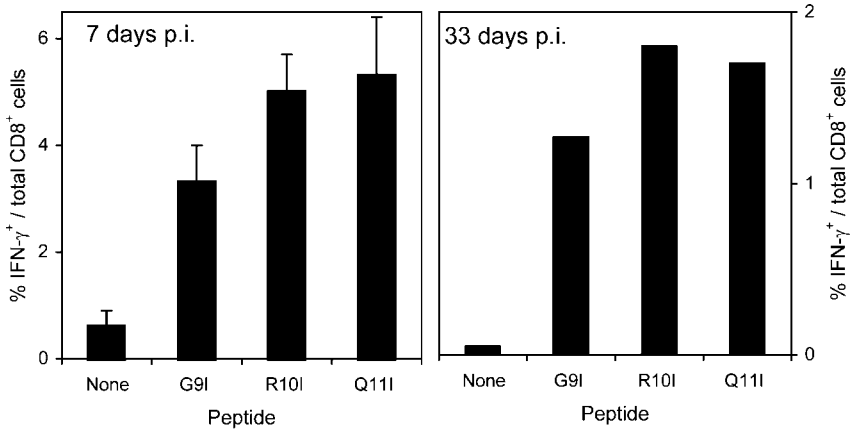

FIG. 8. ***Ex vivo* populations of CD8$^+$ T lymphocytes responsive to synthetic ENV peptides.** Splenocytes from rVV-ENV-infected mice were tested in an ICS assay either after primary infection (7 days postinfection, *left panel*) or in the memory phase (33 days postinfection, *right panel*) with ENV synthetic peptides. Percentages of total CD8$^+$ lymphocytes that stained for IFN-γ after stimulation with the indicated peptides are shown. Data are the mean of 5 mice from three independent experiments for the 7-day (*left panel*) and one representative experiment for the memory (*right panel*).

some tri-charged peptides (Table I, 3 charges) also co-eluted with the second antigenic peak of the cation-exchange column. The shortest example of such peptides, all of which are longer than 13 residues and have bi-terminal extensions of the 10mer core, is I14K (Table I). However, failure to co-elute with the antigenic peak in RP-HPLC excluded them as candidate natural peptides.

Further characterization of the bi-charged candidate synthetic peptides (Fig. 7*A*) shows that ENV-specific CTL similarly and efficiently recognize both R10I and Q11I. Previous reports of lack of antigenicity of the Q11I peptide were ascribed to cyclization of the N-terminal Gln (20), which was excluded in our purified peptide by mass spectrometry. Potential contamination with strongly antigenic G9I was discarded, as purification of our Q11I peptide was performed by cation-exchange chromatography. Contamination of Q11I with a more antigenic species, specifically with R10I, was similarly excluded by mass spectrometry (data not shown). Notably, Fig. 7*A* also shows that the mono-charged G9I peptide identified above presents identical titration curve as both bi-charged peptides. This was true for CTL lines established by *in vitro* restimulation with either G9I or R10I peptides (data not shown). Next, MHC/peptide complex stability analyses were conducted with the synthetic peptides corresponding to the identified mono-charged natural species G9I and with the two candidate bi-charged species R10I and Q11I. Fig. 7*B* shows that the stability of the D^d/G9I complexes was lower, which might be explained by its loss of an anchor position (see "Discussion"). But, remarkably, the results shown in Fig. 7*B* indicated similar rates of complex formation in all three cases (time point 0). Provided similar intracellular pools of each peptide are available, this is compatible with either of the three peptides having the same chance of being selected for binding to MHC class I from a pool of different naturally processed peptides available intracellularly.

To study in depth the *in vivo* relevance of these nested natural D^d ligands, both primary CD8$^+$ responses at 7 days postinfection and memory CD8$^+$ responses at 33 days postinfection were quantitated by ICS. This method provides a more direct measure of CD8$^+$ T lymphocyte priming than does measuring lytic activity of secondary *in vitro* CTL cultures. As shown in Fig. 8, all three peptides induced secretion of IFNγ by activated CD8$^+$ cells. Peptide G9I-recognizing CD8$^+$ T lymphocytes were slightly less abundant that those reacting to the R10I or Q11I peptides, both in the primary and in the memory situation, fully in agreement with the slightly lower stability of D^d/G9I complexes as compared with D^d/R10I or D^d/Q11I (Fig. 7*B*).

Recognition by CTL of serial dilutions of the two antigenic peaks from the ion-exchange chromatography revealed that both activities were present in approximately similar amounts in the original infected cells (Fig. 3). As all three candidate peptides bound equally well to D^d and were also recognized similarly by CTL, it was concluded that the pool of D^d-bound natural peptides in ENV-expressing infected cells consisted of similar amounts of the G9I peptide and of either the R10I or the Q11I peptide, or both.

The Shorter Peptidic Species Is Not Generated from the Longer Ones by the Extraction Procedure—As the two bi-charged candidate natural peptides Q11I and R10I contain and are longer than the monocharged G9I peptide, the latter one could potentially be artificially generated from the former two peptides by an aminopeptidase activity or by a chemical reaction during the biochemical isolation of naturally processed peptides. To exclude this possibility, similar extraction experiments as those performed with rVV-ENV-infected cells were carried out with R10I and Q11I-pulsed D^d-positive target cells. Following RP- and cation-exchange HPLC, collected fractions from this control experiment were tested with CTL and analyzed by mass spectrometry. No production (less than 6%, as calculated from the detection limit of the CTL assay) of G9I peptide, or of any mono-charged antigenic species, was found in these assays (Fig. 9*A*). No evidence of generation of R10I from the longer Q11I, or of cyclization of the N-terminal Q during extract preparation was observed either by mass spectrometry (Fig. 9*B*). These data thus confirm the endogenous generation of G9I in rVV-ENV-infected cells. Additionally, it was confirmed that the R10I and Q11I peptides were not modified by the biochemical extraction, reinforcing their identification as candidate natural peptides in infected cells.

In conclusion, these results indicate that the endogenous processing of the envelope glycoprotein of the HIV-1 strain IIIB generates, at least, two different molecular species that are bound to the presenting molecule in the infected cell. Of these, at least one of them is different to the decamer peptide previously assumed (20) as the optimal synthetic peptide in the ENV glycoprotein-specific CTL immune response, and unexpectedly lacks the terminal NH_3^+ group anchor.

Effect of N- and C-terminal Extensions on MHC/Peptide/CTL Interaction—Resolution of the crystal structure of the R10I peptide bound to D^d (38, 39), as well as analysis of the pool of natural endogenous peptides presented by D^d (40), jointly provide a basis for predicting the binding conformation of the shorter as well as maybe longer peptides found in this study. To complement this set of information, antigenicity for CTL, as well as D^d/peptide complex stability, was assayed for an extended series of synthetic peptides. As shown in Table I, single or double C-terminal extensions of the R10I core had a moderate negative effect on antigenicity, while single or double extensions at the N terminus were still as efficiently recognized by CTL as the optimal peptide. Further N-terminal extensions only very gradually led to decreased antigenicity. Specifically, a

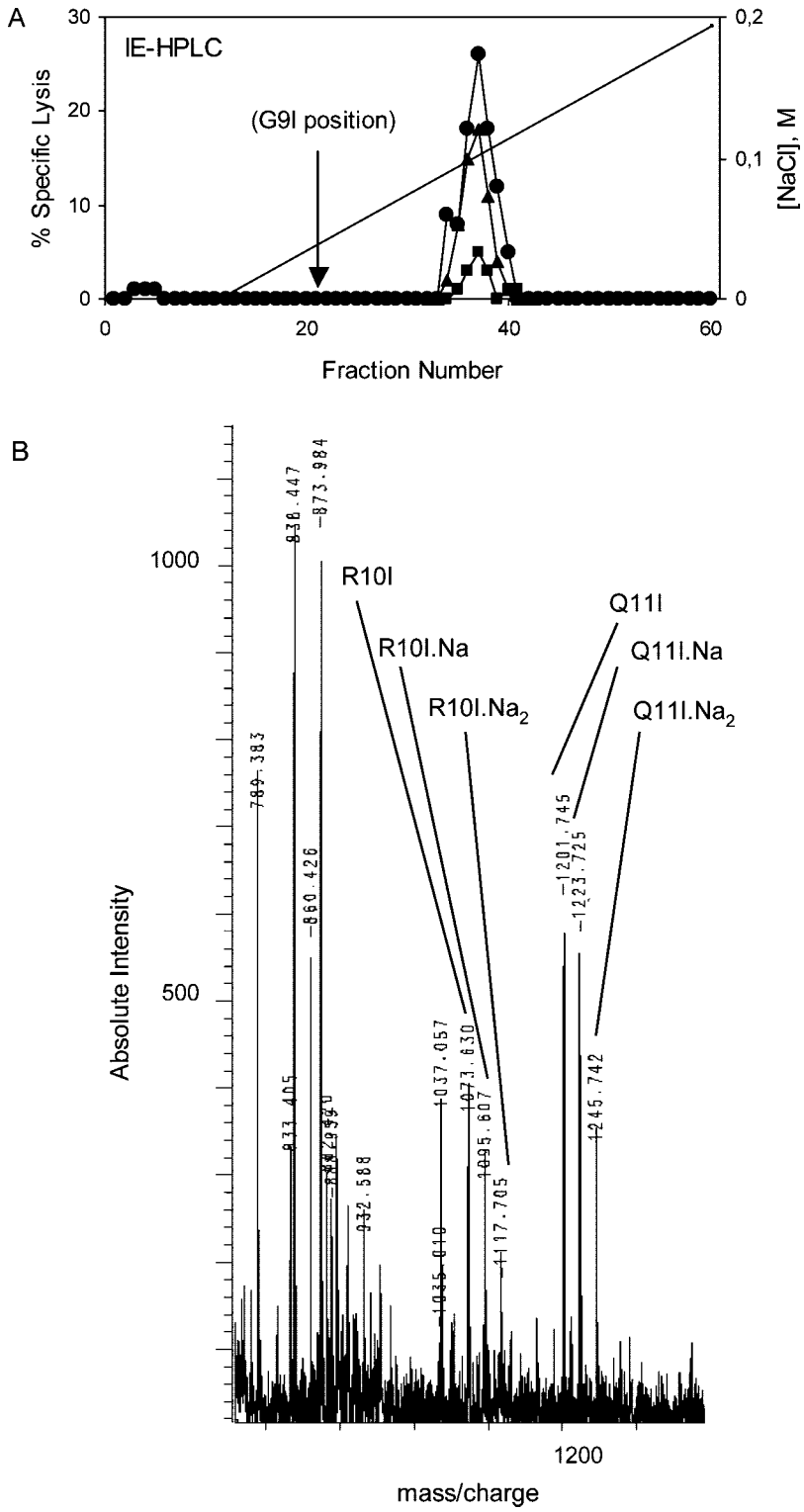

FIG. 9. **Control extracts of uninfected D^d-positive cells with synthetic peptides.** Peptides were extracted from uninfected P13.1 cells to which synthetic Q11I and R10I had been added prior to extraction, and separated by RP-HPLC. Antigenic fractions were selected, further separated by cation-exchange chromatography (IE-HPLC) and tested again with CTL (*panel A*). The results obtained with 1.2 (●), 0.15 (▲), and 0.04 (■) initial nmol of either synthetic peptide in the control extracts are plotted. *Panel B* shows the MALDI-TOF mass spectrometry spectrum of antigenic fraction 37 of the cation-exchange chromatography. A mass/charge ratio of 1073.63 corresponds to the R10I peptide, and that of 1201.74 to Q11I.

three-residue N-terminal extension in R13I was still as antigenic as a single-residue carboxyl extension in R11G. These results are essentially compatible with partial data presented by other groups with some of these peptides (18, 20), before endogenous natural peptides were identified in our present report.

Analysis of a second set of peptides in Table I reveals that removal of the N-terminal amino acid R, as in peptide G9I, did not have a significantly deleterious effect. This is coherent with the lack of effect of N-terminal extensions observed above. However, in this G9I context, combination with C-terminal extensions had a very drastic effect. Similarly, a third set of peptides with combined N- and C-terminal extensions were barely antigenic, starting already with Q12G. This is in full agreement with the described cumulative effect of combined N- and C-terminal alterations on peptide antigenicity (41, 42), while single alterations were fully tolerated (41).

Collectively, this set of data reveals a remarkable tolerance of D^d and CTL for N-terminal alterations of the 10-mer epitope, unprecedented for other MHC class I complexes, a more deleterious effect of C-terminal elongations, while the alteration of both termini essentially abrogates functionality.

DISCUSSION

The results reported here demonstrate that the endogenous processing of the HIV-1 envelope glycoprotein generates, at least, two different peptidic species that are bound to and presented by the H-2D^d molecule in infected cells. They are the nonamer G9I (^{319}GPGRAFVTI^{327}) and either its N-terminal extension R10I (^{318}RGPGRAFVTI^{327}) or its N-terminal double extension Q11I (^{317}QRGPGRAFVTI^{327}). All three peptides induce high numbers of MHC/peptide complexes and are efficiently presented to CTL. To our knowledge, this is the first report of naturally processed peptides derived from HIV ENV protein, and the first time that a naturally processed MHC-bound ligand derived from an HIV protein is unequivocally identified by mass spectrometry. Furthermore, it is the first description of an N-terminally-extended nested set of equally abundant and antigenic natural peptides bound to MHC class I molecules. The unexpected existence of two to three peptidic variants recognized by CTL with the same antigenicity indicates the complexity of antigen presentation, which is sometimes minimized in epitope-detection assays with synthetic peptides.

Previous analysis of synthetic peptides described the R10I peptide as the optimal ENV peptide for D^d (17–21). While intracellular selection by MHC class I molecules of naturally processed peptides frequently coincides with the optimal synthetic peptide, this is by no means the rule. There are several studies that show that the natural peptide derived from viral proteins (43, 44) does not match that suggested from synthetic peptide recognition analysis (45, 46). Other reports reveal a more complex nested set of natural viral peptides that are presented by the same MHC class I molecule, and that comprise very weakly antigenic C-terminal extensions of the antigenically optimal core identified with synthetic peptides (15, 47). Our study also reveals that the natural situation is more complex than that deduced from studies with synthetic peptides, and presents the first example of N-terminal variations of a natural viral peptide. As a crucial novelty compared with previous reports (15, 47), all HIV ENV natural peptide/MHC complexes identified by us are expected to contribute significantly to the physiological CTL response in the infected mouse, as all of them are equally abundant and antigenic, and similar numbers of specific CTL are found for each peptide *in vivo*. All these results show the importance of the analysis of natural peptides that result from the endogenous processing of viral proteins, as this is fundamental for a detailed understanding of MHC class I-restricted immunity. Extension of these results in the mouse model to studies with patient CTL will contribute to vaccine design.

A striking exception was found with the N-terminally extended I12I peptide, which was as antigenic and as efficient in forming stable complexes with D^d as the other three MHC peptidic ligands, Q11I, R10I, and G9I (Table I). Simple experiments with synthetic peptides would have qualified it as a very probable candidate natural peptide. And yet, because its elution profile in the cation-exchange HPLC column corresponded to fraction 30, devoid of antigenicity in the infected cell extracts, it was concluded that it was not present among the pool of naturally processed peptides. Because the evidence indicates that intracellular D^d would bind this peptide if given the chance, this implies that this I12I peptide may not be produced by the proteasome. Alternatively, it might be rapidly converted into shorter variants before it binds, or after binding (48) to D^d. Thus, the I12I peptide represents a paradigm where analysis of natural endogenous peptides is critically required to unequivocally establish the physiological relevance of a very antigenic synthetic peptide.

Concerning transport by TAP, peptides I12I and Q11I, as well as potential longer precursors, would not have any theoretical limitations. However, transport of the decamer R10I and of the nonamer G9I would be disfavored by murine TAP, because they have a P at position 3 and 2, respectively (49–51). Thus, G9I, and R10I if confirmed as a natural peptide in murine cells, would be generated from a longer precursor that is efficiently transported into the endoplasmic reticulum. Among other possibilities, this precursor might well be the Q11I peptide itself.

Once in the secretory pathway, either endoproteases or aminopeptidases should produce the proximal N-terminal cleavages to yield the final peptides. Specifically, a crucial protease should be an uncharacterized metallopeptidase that operates in a sequential pathway with the proteasomes to process ENV (28). In the endoplasmic reticulum, a metallo-aminopeptidase has been described that leads to the accumulation of peptides with P at position 2 (52). This aminopeptidase could be responsible for the production of G9I, and a potential accumulation of this peptide would result in its over-representation in the pool of natural peptides, despite the lower stability of the D^d/G9I complexes. This aminopeptidase, or others (53, 54, 59), could also sequentially have generated the undecamer and the decamer, although it is also possible that these peptides are generated individually from longer precursors by endo- or tripeptidyl- or dipeptidyl-aminopeptidases (53).

Whereas most MHC class I molecules require two major anchor residues in the antigenic peptide, the D^d allele has an unusual binding motif with three major anchors (Gly at position 2, Pro at 3, and Leu, Ile, or Phe at the C-terminal position) and a positively charged residue at position 5 (40). This fourth anchor residue is due to the presence of two Trp residues (at positions 97 and 114 of D^d), which form a wall within the binding groove that forces the peptide to arch over the wall to anchor the C-terminal residue (39), and permits that peptide positions 6, 7, and 8 protrude above the protein surface and have high solvent accessibility. As a consequence, the side chains of these residues are more accessible for recognition by specific CTL. Crystallographic studies (38, 39) show that the R10I peptide binds to the presenting molecule D^d with the lateral chain of the C-terminal residue anchored in the F pocket. The NH_3^+ group of the N-terminal Arg binds to the A pocket, while its lateral chain points out of the groove. Positions 2G, 3P, 5R, and 10I are deeply buried in the groove, and confer a more constrained conformation to R10I than that of peptides bound to other MHC alleles. Collectively, the data suggest that the nonamer G9I binds to D^d in a very similar conformation as R10I does, with each side chain occupying a similar position as those of R10I in the elucidated D^d/R10I complex. The only and important difference between both D^d-bound peptides would be the lack of interaction of the terminal NH_3^+ group of G9I with the A pocket residues. This model (Fig. 10) for G9I binding to D^d accounts for the identical antigenic recognition of both G9I and R10I peptides by CTL despite the weaker stability of the D^d/G9I complexes. Furthermore, the lack of strict requirement for the NH_3^+ group interaction is supported by the striking lack of effect of its loss in peptides elongated several amino acids on the N terminus (Table I).

As the R10I peptide is highly constrained by four anchor motifs and position 1 protrudes above the surface of the D^d molecule, the simplest possibility is that the additional amino-extended residue 1Q of the Q11I peptide reaches out of the peptide cleft (Fig. 10) without altering the peptide surface exposed to TCR recognition. This is compatible with peptides G9I, R10I, and Q11I showing similar complex formation rates and identical recognition curves by specific CTL. In addition,

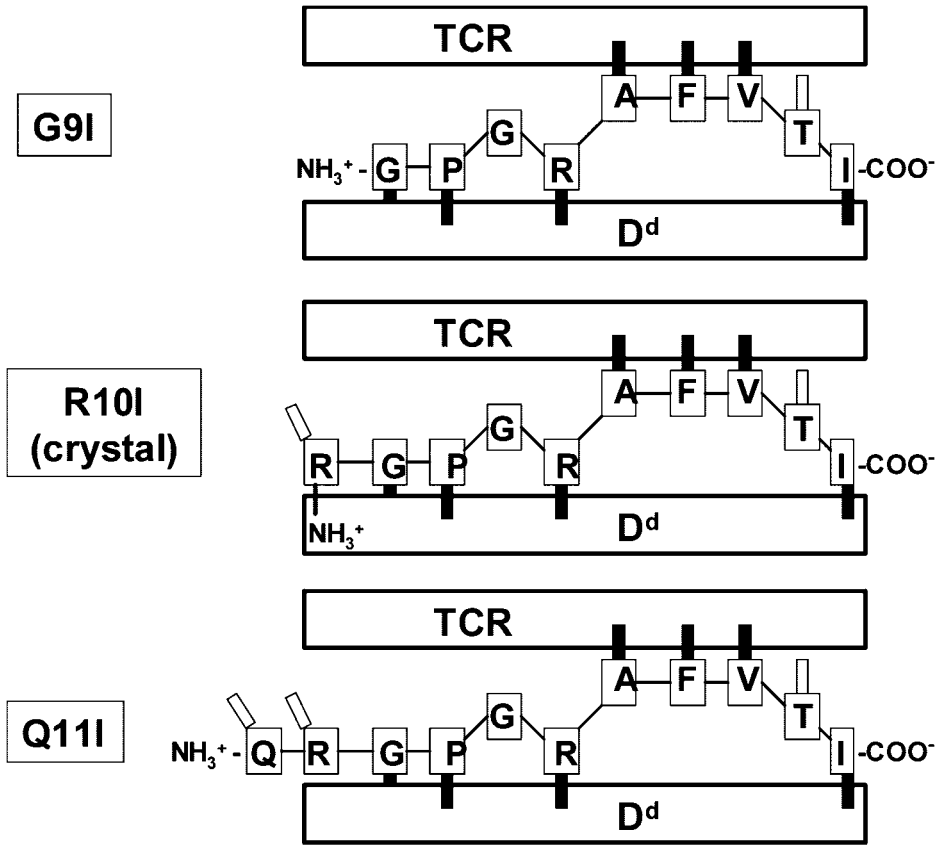

FIG. 10. **A model for binding of peptides G9I and Q11I to D^d.** The model for the G9I/D^d and the Q11I/D^d complexes is based on the crystal structure of the R10I/D^d complex (38, 39) and the results reported in this article. Side chains of peptide residues are represented by *filled rectangles* to indicate interaction with D^d or exposure to the T-cell receptor, TCR. *Void rectangles* indicate lower or no interaction. The main chain of the most N-terminal Gly is similarly represented with a *filled small square* where it interacts with D^d.

this conclusion is further reinforced by the observation that the 12-mer N-terminal elongated peptide I12I was also as efficiently recognized by the same specific CTL, and as efficient in complex formation with D^d, and by the fact that peptides with longer N-terminal extensions were still remarkably antigenic. In contrast, C-terminal extensions are disfavored, maybe because position 10I is deeply buried into the hydrophobic F pocket and allows for little flexibility.

The occurrence of G9I or Q11I as natural peptides would not have been predicted, however, from analysis of the cellular peptides eluted from D^d molecules (40). Only 9 or 10 amino acid-long peptides with both NH_3^+ and carboxyl anchors were identified, that differed only in the degree of central bulging. This is the most frequent structural adaptation for accommodating longer peptides by MHC class I molecules. Binding of G9I without an NH_3^+ group anchor, as indicated by our evidence, is supported by the existence of peptides lacking the N-terminal binding residue that nevertheless bind endogenously to other MHC class I molecules (55), which indicates that canonical MHC-peptide interactions in the A pocket are not always necessary for endogenous peptide presentation.

Regarding Q11I, if confirmed, it would represent just the second report of an N-terminal extended natural peptide, and the first case where the MHC/peptide complex is abundant and antigenic enough to contribute to the physiological CTL response. Indeed, in the previously described example, the amount and the antigenicity of the complexes with the amino-extended precursor were both 100-fold lower than those of the final core peptide (48). In contrast, several instances of longer than canonical endogenous peptides that very likely protrude on the C terminus have been reported (15, 47, 56–58), although comparable antigenicity to the core epitope has not been demonstrated for any of them. A likely explanation for this bias toward carboxyl extensions is the existence of aminopeptidases, but not of carboxypeptidases, in the proximal part of the secretory route, where most MHC/peptide complexes are formed. These aminopeptidases might trim (48) a fraction of the Q11I/D^d complexes and reduce their relative presence at the plasma membrane, increasing the numbers of surface R10I and/or G9I complexes.

The diversity of MHC/peptide complexes can be an advantage for the immune system. First, a wider variety of complexes can activate a wider repertoire of T-cell clonotypes, generating a more solid CTL response. Even if the residues shared by all natural peptides bind similarly to D^d, additional extending residues can individually contribute to recognition by the TCR (42). Second, one advantage of this richness of MHC/peptide complexes is that none of them is strictly necessary to generate a response. For example, although Q11I is a very good candidate natural precursor or ligand, it is not a necessary molecule, as the eC-10env full-length chimeric protein that lacks the N-terminal Gln can nevertheless be processed for presentation by D^d (28). Thus, the existence of multiple MHC/peptide complexes can deaden the negative effects of antigen variability and reduce the likelihood of appearance of CTL escape variants (1, 10, 11). It is a paradox that a hypervariable region of HIV ENV protein, as the V3 loop, is nevertheless such a good source of epitopes from many different HIV strains and in a high number of patients. Our results contribute to an explanation. Apparently, several different peptides can be processed from this area. From this pool, a single MHC class I molecule, D^d, already can sample two or three different peptides to complex with. Even after natural variation of the epitope environment in the viral protein, that may preclude the generation of individual species, some of the peptides would still be processed, still allowing for antigen presentation and thus for the generation of an efficient CTL immune response. A third advantage of generating a pool of processed peptides is that it can allow presentation of related but distinct peptides by a wider number of different MHC class I alleles, as indeed is the case for this

epitopic area (22), providing a more extended immunity to the population.

Identification of the natural peptides presented by further murine and human MHC class I molecules is needed, not only to confirm this hypothesis, but also to learn more about the general rules of antigen processing, and specifically in this antigenic area. In addition, the knowledge obtained from these and future studies on endogenous peptides can help prevent biases in vaccine research. Clinical trials have started with recombinant constructs containing multiple minimal epitopes linked in a string. With the limited knowledge uncovered by our experiments in the murine model, we can already propose that a larger area, including Q11I and possibly extended to the unknown sequences that allow the generation of multiple processed products, should be included in any vaccine formulation aiming at inducing a solid CTL immunity to the HIV envelope glycoprotein in several HLA backgrounds.

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