Cytotoxic T Lymphocyte Epitopes of HIV-1 Nef: 
Generation of Multiple Definitive Major Histocompatibility 
Complex Class I Ligands by Proteasomes

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Abstract
Although a pivotal role of proteasomes in the proteolytic generation of epitopes for major histocompatibility complex (MHC) class I presentation is undisputed, their precise function is currently the subject of an active debate: do proteasomes generate many epitopes in definitive form, or do they merely generate the COOH termini, whereas the definitive NH2 termini are cleaved by aminopeptidases? We determined five naturally processed MHC class I ligands derived from HIV-1 Nef. Unexpectedly, the five ligands correspond to only three cytotoxic T lymphocyte (CTL) epitopes, two of which occur in two COOH-terminal length variants. Parallel analyses of proteasomal digests of a Nef fragment encompassing the epitopes revealed that all five ligands are direct products of proteasomes. Moreover, in four of the five ligands, the NH2 termini correspond to major proteasome cleavage sites, and putative NH2-terminally extended precursor fragments were detected for only one of the five ligands. All ligands are transported by the transporter associated with antigen processing (TAP). The combined results from these five ligands provide strong evidence that many definitive MHC class I ligands are precisely cleaved at both ends by proteasomes. Additional evidence supporting this conclusion is discussed, along with contrasting results of others who propose a strong role for NH2-terminal trimming with direct proteasomal epitope generation being a rare event.

Key words: proteasome • HIV Nef • cytotoxic T lymphocyte epitopes • antigen processing • naturally processed peptides

Introduction
Proteasomes are the major cytosolic proteases of eukaryotic cells and participate in the processing of many antigens presented by MHC class I molecules. However, the precise role of proteasomes in epitope generation is not yet clear. It is particularly a matter of debate whether proteasomes often generate both COOH and NH2 termini of proteasome-dependent epitopes, or whether they only generate their COOH termini (1, 2).

Purified proteasomes degrade polypeptides into a large number of extensively overlapping oligopeptides. Most major peptide products of vertebrate 20S proteasomes have hydrophobic, acidic, or basic amino acids (aa)1 at the COOH terminus, most frequently leucine. Peptides with other aa at the COOH terminus are produced less frequently and are usually found in small amounts. With the exception of acidic residues, which are found in proteasome products but very rarely in MHC class I ligands, a similar distribution of aa is observed at the COOH termini of MHC class I ligands. Small neutral and polar aa, especially serine, are enriched at the NH2 termini of proteasome products.
eral products, also similar to MHC class I ligands (3–5). With a few exceptions, the size range of peptides eluted from typical MHC class I molecules is 7–13 aa, and most alleles prefer nonamers (6). Although some peptides produced by vertebrate 205 or 265 proteasomes from polypeptides or proteins are shorter than 8 aa, only a few are larger than 11 aa (3, 4, 7), and many major products are in the size range of MHC class I ligands (3, 4). Digestion of relatively short polypeptides proceeds partially via single cleavage intermediates, formation of “dual cleavage” peptides, i.e., MHC class I ligands and slightly longer peptides, is accelerated by the IFN-γ-inducible proteasome activator 28 (PA28 [4, 8]). Degradation of most full-length proteins is progressive, without the release of longer intermediates (9, 10). Of 10 MHC class I ligands examined so far in various laboratories for production by purified proteasomes in vitro, 7 were shown to be generated in definitive form (see Discussion). Collectively, these data support the conclusion that proteasomes produce both the COOH and NH₂ termini of many MHC class I ligands in vitro.

Nevertheless, these data have not led to a general consensus on the precise role of proteasomes in antigen processing. The validity of the in vitro results has been challenged by observations on the generation of the major OVA epitope SIINFEKL from the products of minigenes (11). Although the production of the epitope from COOH-terminally (Ct)-extended versions was inhibited by the proteasome inhibitor lactacystin, production from NH₂-terminally (Nt)-extended versions was not. Moreover, SIINFEKL could be produced from the Nt-extended fragment QLLESINFEKL by leucine aminopeptidase (LAP), a cytosolic peptidase inducible by IFN-γ (12). This was put forward as evidence that the SIINFEKL NH₂ terminus is produced in vivo by trimming enzymes rather than by proteasomes. Although these results can be interpreted in alternative ways (see Discussion), they have led to the general perception that proteasomes mainly release the COOH termini of class I ligands, whereas the NH₂ termini are derived by trimming of longer precursors by aminopeptidases (1, 13).

Nef is a key factor in HIV pathogenicity and immunogenicity and a potential candidate for CTL-targeted vaccination. More than 45 CTL epitopes for multiple HLA alleles have been described in HIV Nef by the use of overlapping synthetic peptides and/or “allele-specific” peptide motifs (14). Many of the Nef CTL epitopes are overlapping, and most of them cluster within four regions of the protein. To date, naturally processed peptides corresponding to these epitopes have not been determined. In this study we focused on one of the four immunogenic regions of Nef, namely the NH₂-terminus of Nef (123–152) which is relatively conserved among different HIV subtypes (15). We identified two HLA-A2 and three HLA-B7 peptide ligands in this region. Unexpectedly, both HLA-A2 peptides and two of the three HLA-B7 ligands represented COOH-terminal length variants of one HLA-A2 and one HLA-B7 epitope, respectively. Proteolytic fragments identical to each of the five definitive MHC class I ligands were found in proteasomal digests of the synthetic polypeptide Nef (123–152). In four of these five naturally processed peptides, the NH₂ 123–152 were produced by major proteasome cleavage sites, and Nt-extended precursors were found for only one of the five peptides. Thus, at least four of the class I ligands are loaded onto HLA molecules in the form generated by proteasomes. These results favor the view that proteasomes often produce the peptides finally presented by MHC class I molecules.

Materials and Methods

Cell Lines. The human lymphoblastoid T cell lines T1 (HLA class I typing A2, B5; subtyping for A2 is A*0201) and Jurkat (HLA I typing A9/25, B7/41; subtyping for B7 is B*0702), the HLA-A– and HLA-B–deficient C1R human lymphoblastoid B cell lines transfected with HLA-A2 or HLA-B7 (C1R-A2 and C1R-B7, respectively), and the P815 murine mastocytoma cell lines transfected with HLA-A2 or HLA-B7 (P815-A2 and P815-B7, respectively), have been described elsewhere (16–19). C1R-A2 and C1R-B7 cells were stably transfected with the pcF+ EBV vector containing the sequence encoding the whole Nef protein from the HIV-1 strain LAI under the control of the cytomegalovirus promoter (20). These cells, referred to as Nef⁺ C1R-A2 and Nef⁻ C1R-B7, were used as stimulator cells to induce polyclonal Nef-specific CTL lines and as target cells in experiments with proteasome inhibitors. We were not able to maintain large scale cultures of C1R-A2 and C1R-B7 cells stably expressing Nef for acid elution of naturally processed peptides. For this purpose, we used T1 and Jurkat cells transfected with the heavy metal-inducible vector pSBBR U6 containing the sequence encoding the whole Nef protein from HIV-1 strain LAI, under the control of the human metallothionein II A promoter (21). These cells are referred to as Nef⁺ T1 cells and Nef⁻ Jurkat cells. Expression of Nef in the transfectants was verified by Western blot analysis. P815-A2 and P815-B7 cells were used as target cells pulsed with peptides in cytotoxicity assays because C1R cells had a high spontaneous ⁵¹Cr release. The human T1 line was used for isolation of proteasomes.

In Vitro Induction of Primary Nef-specific CTL Lines. Polyclonal Nef-specific CTL lines were induced as described (20) using PBLs of a healthy HIV-1 seronegative donor (HLA class I typing A2/28, B7/40w60, Cw3/w7; A2 and B7 subtypes are A*0201 and B*0702, respectively) and Nef⁺ C1R-A2 or Nef⁻ C1R-B7 as stimulator cells. Nef peptide-specific CTL lines were obtained from the same donor (22). In brief, PBLs (4 or 5 × 10⁶) were incubated in 24-well culture plates (Nalge Nunc International) with RPMI 1640 (GIBCO BRL) supplemented with 5% human AB serum (Blood Donor Center Schweizerisches Rotes Kreuz [SRK], Basel, Switzerland), 1 mM sodium pyruvate, 20 mM Hepes, 2 mM glutamine, 100 U/100 µl penicillin/streptomycin (all from GIBCO BRL), 1 × 10⁻⁵ M 2-ME (Sigma Chemical Co.), and 1% MEM nonessential amino acids (GIBCO BRL) for 90 min at 37°C. The nonadherent cells were removed, and the adherent fraction (monocytes) was pulsed with 100 µg/ml of peptide for 4 h to be used as stimulator cells. Adherent cells were then incubated with 1 x 10⁶ autologous PBLs. After 5 d of culture, 100 U/ml proleukin (Chiron Corp.) was added. After an additional 7 d, the PBLs were restimulated with 1 x 10⁶ irradiated autologous PHA (Murex Biotech, Ltd.) T cell blasts, pulsed with 10 µg/ml of peptide.

Cytotoxicity Assay. Cytolytic activity was tested in triplicate in a standard 4-h ⁵¹Cr-release assay (23). Activity was assayed against cells expressing Nef as well as against P815-A2 and P815-B7 cells pulsed with synthetic peptides or reversed phase HPLC.
Digests were incubated in a total volume of 300 μl buffer consisting of 15 mM cold 1% TFA (Sigma Chemical Co.) and disrupted using a hand-held glass homogenizer. Centriprep 10K (Amicon Corp.) was used for fractionation by rHPLC. Amino acid residues are given in single-letter code as follows: C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Extraction of Naturally Processed Peptides. Naturally processed peptides were extracted from whole cells with TFA (25) and purified as described previously (26, 27). Batches of 10^6 Nef^+ T1, Nef^+ Jurkat, and Nef^- Jurkat cells were lysed by adding 15 ml cold 1% TFA (Sigma Chemical Co.) and disrupted using a hand-held glass homogenizer. Centriprep 10K (Amicon Corp.) centrifugal concentrators were used for isolation of low molecular weight peptides. The ultrafiltrate suspensions (molecular weight <10 kD) were dried by lyophilization and redissolved in 0.1% TFA for fractionation by rHPLC.

In Vitro Digestion of Polyepitope Substrate by Purified 20S Proteasomes. 20S proteasomes were purified from human T1 cells as described previously (4). No impurities were detected by SDS or native gel electrophoresis. Trypsin-potent peptides II (TPII) contaminations were excluded by use of the TPII inhibitors AAF-chloromethylketone (Bachem) and PM SF (Sigma Chemical Co.). Digestion of the synthetic 30-mer peptide Nef_123-152 (10 μg) with isolated 20S proteasomes (2 μg) was carried out at 37°C. Digests were incubated in a total volume of 300 μl buffer consisting of 50 mM Tris-HCl (pH 7.8), 1 mM EGTA, 0.5 mM EDTA, 5 mM Mgc2, 0.5 mM 2-ME, and 0.02% azide. Aliquots of the reaction mixture were fractionated by rHPLC.

rHPLC Frationation and Analysis of Naturally and Synthetic Peptides. Aliquots of rHPLC fractions were separated by rHPLC (Smart System [Amersham Pharmacia Biotech]) equipped with a Sephasil C18 SC2.1/10 column) with the following: eluent A, 0.1% TFA; eluent B, 80% acetonitrile containing 0.081% TFA; flow rate, 100 μl/min; gradient for separation of HLA-A2-restricted peptides, 34.3–35.5% B in 55 min (increase of eluent B, 0.022% per min); gradient for separation of HLA-B7-restricted peptides, 23.5–24.4% B in 28 min (increase of eluent B, 0.032% per min) followed by 24.4–26.5% B in 27 min (increase of 0.078% per min). Fractions were collected from 30 to 85 min (fractions 1–59), and elution was monitored by measuring UV light absorption at 214 nm in a continuous flow detector. TFA/acetonitrile ratio was calculated as the mean fluorescence of the sample versus the mean fluorescence of the control. The synthetic reference peptide used was the HLA-B7–restricted self-peptide APRTVALTAL. The peptide Nef_198-217, which does not bind to HLA-B7, was used as a negative control.

Peptide affinity for immunity-affinity-purified HLA-A2 molecules was measured in a competitive binding assay based on published procedures (30). In brief, HLA-A2 molecules were purified from NP-40 lysates of the homozygous B cell line Jhestorn using Sephasore-immobilized BB7.2 (anti-A2) mAb. Purified HLA-A2 molecules (400 ng) were incubated for 18 h with 2.4 pmol of iodinated reporter peptide hepatitis B virus (HBV) core 18-27 (6Y) and various concentrations of unlabelled competitor peptides in a total volume of 30 μl PBS buffer with 0.05% NP-40 and 1 mM PM SF. Peptide binding was stopped and evaluated by a 4-min centrifugation at 25°C and 1,000 g through gel filtration columns (Micro Bio-Spin 30: Bio-Rad Laboratories). Bound peptide was quantified by gamma counting of filtrates. The results are expressed as relative IC_{50} and are mean values from three experiments.

Quantitation of HLA Class I Ligands in Nef-Transfected Cells. The molar concentrations of HLA ligands in rHPLC fractions of acid eluates of naturally transfected cells were determined by titration of rHPLC fractions in a 4-h 3HCr-release assay, and comparison of the percent specific lysis was obtained with a standard curve of known concentrations of synthetic peptide. The molar amounts of peptide ligands obtained per extraction were multiplied by aAvogadro’s number and divided by the number of cells that were extracted. Recoveries of control synthetic peptides were determined as described (26).

Results

Effect of Proteasome Inhibitors on Intracellular Nef Processing. Lactacystin is an efficient inhibitor of the chymotrypsin- and trypsin-like activities of proteasomes (31), and a weaker inhibitor of the cytoplasmic proteasome complex TPII (32). To assess whether proteasomes are involved in the processing of Nef, we studied the effect of lactacystin on the HLA-A2- and HLA-B7-restricted presentation of Nef, using concentrations that discriminate between proteasomes and TPII. Nef^-^C1R-A2 and Nef^-^C1R-B7 cells were incubated with test peptides. The concentrations required for 50% inhibition of specific binding (IC_{50}) were normalized with respect to the IC_{50} of the reporter peptide (typically ~400 nM). The results are expressed as relative IC_{50} and are mean values from three experiments.
bated for 2 h in the presence of 10 μM lactacystin and then briefly exposed to pH 3.1 to denature and remove surface class I peptide complexes. Acid-stripped target cells were then allowed to reexpress MHC class I–peptide complexes for 4 h during a standard 51Cr-release assay, in the presence or absence of 10 μM lactacystin. In the absence of lactacystin, target cell lysis by HLA-A2– and HLA-B7–restricted Nef-specific CTLs was restored after acid treatment (Fig. 1), reaching 80–100% of the lysis of untreated target cells (data not shown). Incubation of acid-treated cells with lactacystin completely abrogated the restoration of HLA-A2– and HLA-B7–restricted CTL recognition of Nef epitopes (Fig. 1). CTL recognition of acid- and lactacystin-treated target cells was restored by addition of known HLA-A2– or HLA-B7–binding Nef peptides, excluding nonspecific deterioration of target cells or of CTLs by the experimental procedures (Fig. 1). A similar degree of inhibition was observed with the peptide aldehyde inhibitor N-acetyl-leucinyl-leucinyl-norleucinal, another potent but less specific proteasome inhibitor (data not shown). These results suggested that processing of Nef for presentation by HLA-A2 as well as HLA-B7 MHC molecules was dependent on proteasomes.

**Design of the Experiments.** One of the four immunogenic regions of Nef (Nef123–152, Table I) was chosen for this study. One HLA-A2 (Nef136PLTFGWCYKL145) and two HLA-B7 (Nef128TPGPGVRYPL137 and Nef135YPLTFGWCY143) restricted CTL epitopes in this region have been identified previously, using polyclonal Nef-specific CTLs from HIV-seropositive donors and target cells pulsed with overlapping Nef peptides or Nef epitopes predicted according to allele-specific motifs (34, 35, 37). We now wanted to determine the naturally processed peptides corresponding to these epitopes. For acid elution from Nef-transfected cells, large-scale cell cultures of Nef-expressing cells were necessary. Because of the cytotoxic effect of Nef, attempts to prepare large-scale cultures of cells stably transfected with Nef have been 242 Proteasomal Generation of HIV Nef Epitopes

<table>
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<td>NYTPGPGVRYPLT</td>
<td>B7</td>
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<td>B7</td>
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<td>A1, B8, B18, B49</td>
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<td>B57</td>
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<td>B18, B49</td>
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*The single-letter amino acid code is used.
rarely been successful (41, 42). To overcome these problems, we used a regulated Nef expression vector (pSBBR U nef) based on a mutated version of the heavy metal-inducible human metallothionein IIA promoter. T1 (HLA class I typing A2, B5) and Jurkat (HLA I typing A9/25, B7/41) cells transfected with this vector produce low basal levels of Nef compatible with large-scale cultures (21). Several liters of either T1 or Jurkat cells stably transfected with pSBBR U nef were grown, followed by induction of Nef expression for 24 h. Peptides were isolated by acid extraction of cell lysates followed by ultrafiltration (10-kD cutoff).

In parallel, a synthetic polypeptide corresponding to the region Nef123–152 was digested with 20S proteasomes isolated from T1 cells. Both peptide pools, that of the acid-extracted naturally processed peptides and that obtained upon proteasomal digestion of Nef123–152, were separated under the exact same conditions by rp-HPLC on an analytical C18 column. Fractions obtained were tested for their ability to sensitize target cells expressing the appropriate MHC class I restriction elements for recognition by Nef peptide-specific CTL lines. To identify the relevant peptides in fractions recognized by CTLs, retention times were compared with those of a series of synthetic Nef-derived overlapping peptides recognized by the same CTLs (Figs. 2 and 3, arrows). To achieve efficient separation of such closely related peptides, extremely shallow TFA/acetonitrile gradients were used, individually adjusted for the analysis of each of the epitopes under study (see Materials and Methods). To ascertain that peptides identified in acid-eluted fractions were indeed Nef-derived MHC ligands, control lysates of cells

![Figure 2](image_url)

**Figure 2.** Comparison between HLA-A2-restricted naturally processed HIV-1 Nef peptides and products derived from in vitro 20S proteasomal degradation of the synthetic polypeptide HIV-1 Nef123–152. Acid-soluble extracts prepared from Nef T1 cells and peptide products derived from 20S proteasome-mediated degradation of the synthetic 30-mer polypeptide Nef123–152 were fractionated by rp-HPLC using a very shallow TFA/acetonitrile gradient (see Materials and Methods). Individual fractions were tested for their ability to sensitize P815-A2 cells for lysis by CTL lines specific for the peptides Nef136PLTFGWCYKL145 (A) and Nef136PLTFGWCYKLV146 (B) in a 4-h 51Cr-release assay. Acid-soluble extracts from Nef T1 cells fractionated before the eluate of Nef T1 cells (A and B, top left), or mock rp-HPLC fractions (buffer only) collected before fractionation of the proteasomal products (A and B, bottom left), gave no activity. The A2-restricted Nef136PLTFGWCYKL145 peptide-specific CTL line did not lyse P815-A2 cells pulsed with peptides eluted from HLA-A2-restricted Nef123–152 Jurkat cells (A, top left). CTL assays were carried out using an E/T ratio of 50:1 (A and B, left) or at different E/T ratios as indicated (A and B, right). The elution position of synthetic peptides is indicated by arrows. The results are representative of five independent experiments.
Comparison between HLA-A2-restricted naturally processed Nef peptides and peptides derived from proteasomal degradation of the 30-mer Nef Polypeptide. Fractions obtained upon rp-HPLC separation of acid extracts of A2+, Nef+ T1 cells were tested for the ability to sensitize HLA-A2+ target cells for lysis by HLA-A2-restricted CTL lines induced against the peptide Nef_{136}PTFGWCYKLV_{146} (Fig. 2 A, top). Surprisingly, Nef_{136}PTFGWCYKL_{145}-specific CTLs recognized not only fractions coeluting with the synthetic peptide Nef_{136}PTFGWCYKL_{145} (fraction 21/22), but also other fractions coeluting with the synthetic peptide Nef_{136}PTFGWCYKL_{146} as shown with Fig. 2 B (top), these CTLs also recognized fractions containing Nef_{136}PTFGWCYKL_{145} as well as fractions containing Nef_{136}PTFGWCYKL_{146}. Neither CTL line recognized peptide material eluted from Nef− T1 cells (Fig. 2, A [top] and B [top]) or peptides eluted from A2+, Nef− Jurkat cells (Fig. 2 A, top left; data for Nef_{136}PTFGWCYKL_{146}-specific CTLs not shown). These controls indicate that the acid-eluted peptides recognized by both CTL lines were derived from Nef-derived and specifically associated with HLA-A2 molecules of the T1 cells. Moreover and unexpectedly, the results suggest that the two peptides represent two naturally processed versions of the same epitope, differing in length by one COOH-terminal aa.

In parallel, a digest of Nef fragment_{123-152} was prepared with isolated proteasomes, fractionated, and analyzed by the same protocol (Fig. 2, A [bottom] and B [bottom]). Strikingly, both CTL lines recognized the exact same rp-HPLC fractions as in the Nef+ T1 cell extracts (fractions 21/22 and 33–35). Active fractions identified in the Nef+ T1 cell extracts and in the products derived by proteasomal digestion and corresponding control fractions were reexamined with serial dilutions of the CTLs, confirming the single E/T ratio results of individual fractions (Fig. 2, A [right] and B [right]). These results strongly suggested that the HLA-A2-restricted CTL epitopes detected in material eluted from Nef-transfected cells were identical to those epitopes identified in the proteasomal digest.

Comparison between HLA-B7-restricted naturally processed Nef peptides and Peptides derived from proteasomal degradation of the 30-mer Nef Polypeptide. To identify HLA-B7-restricted naturally processed Nef peptides, peptides were acid eluted from B7+, Nef+ Jurkat cells and fractionated by rp-HPLC. Fractions were first screened with an HLA-B7-restricted CTL line generated against Nef_{128}TPPGGVRYL_{137}. Again surprisingly, Nef_{128}TPPGGVRYL_{137}-specific CTLs recognized two rp-HPLC fractions of acid-eluted material: fraction 3 corresponding to the elution time of Nef_{128}TPPGGVRYL_{135}, and fraction 15 corresponding to the elution time of Nef_{128}TPPGGVRYL_{137} (Fig. 3 A, top). Accordingly, CTLs were prepared against the smaller peptide, the octamer Nef_{128}TPPGGVRYL_{135}, and these were found to recognize the same two fractions (Fig. 3 B). These results suggest that the two peptides represent two naturally processed versions of the same epitope, differing in length by two COOH-terminal aa.

Peptides eluted from Nef-transfected Jurkat cells were also screened with a CTL line against Nef_{128}YPLFGWCY_{135}. This CTL line recognized a single fraction of naturally processed peptides (fraction 23), corresponding to the elution time of the inducing peptide (Fig. 3 C, top). This suggests that Nef_{128}YPLFGWCY_{135} is indeed a naturally processed peptide, and that this epitope exists only in one HLA-B7-binding version. However, the epitope overlaps extensively with the HLA-A2-binding naturally processed peptides Nef_{136}PTFGWCYKL_{145} and Nef_{136}PTFGWCYKL_{146} described in the previous section. All three HLA-B7-restricted Nef peptide–specific CTL lines failed to recognize peptide material eluted from Nef− Jurkat cells or HLA-B7−, Nef+ T1 cells, indicating that the peptides eluted from Nef-transfected Jurkat cells were Nef derived and specifically bound to HLA-B7 molecules (Fig. 3 B, top left).

In parallel, proteasomal digests of Nef_{123-152} were separated by rp-HPLC using the exact same gradients. Each of the three HLA-B7-restricted CTL lines recognized fractions identical to that of the acid-eluted peptides (Fig. 3, A–C; compare top and bottom). Active fractions identified in the Nef-transfected Jurkat cell extracts and in the products derived by proteasomal digestion and corresponding control fractions were reexamined with serial dilutions of CTLs, confirming the single E/T ratio results of individual fractions (Fig. 3, A [right] and B [right]). These results suggest that the peptides recognized by the three HLA-B7-restricted CTLs in acid eluates of Nef-transfected Jurkat cells and in proteasomal digests of Nef_{123-152} were identical.

Comprehensive analysis of peptide fragments generated by digestion of Nef_{123-152} with purified proteasomes in vitro. The proteasomal digest of Nef_{123-152} was separated by rp-HPLC using a relatively steep gradient to recover the vast majority of all possible fragments (Fig. 4 A). The peptides produced were identified by mass spectrometry and Edman degradation. Fig. 4 B shows a digestion map compiling all identified degradation products and proteasomal cleavage sites, the strength of the latter according to the quantification of the adjacent products by Edman degradation. All of the five naturally processed peptides detected by CTLs (see above) could also be found by protein analytical methods. The HLA-B7-binding octamer peptide Nef_{128}TPPGGVRYL_{135} is the major dual cleavage product, presumably because it is flanked by predominant cleavage sites and very little cleavage occurs internally. The remaining four peptides suffer more pronounced internal cleavage and are thus produced in smaller, but still considerable amounts. It is remarkable that significant quantities of the HLA-B7 ligands Nef_{128}TPPGGVRYL_{135}, Nef_{135}PTFGWCY_{143} and Nef_{135}TPPGGVRYL_{137} and Nef_{135}PTFGWCY_{143} are produced upon proteasomal digestion, as both peptides are subject to destruction by the predominant cleavage site in the polypeptide Y_{135}-P_{136}. This major cleavage site generates the COOH terminus of the abundant HLA-B7-binding octamer Nef_{128-135} as well as the NH2 termini of the two naturally processed HLA-A2 ligands. Most signifi-
cantly, in four of the five naturally processed peptides (Nef128TPGPGVRY135, 128TPGPGVRYPL137, 136PLTFGWCYKL145, and 136PLTFGWCYKL146), the NH₂ termini correspond to major proteasomal cleavage sites.

Peptide Binding to TAP and MHC Class I Molecules. The results described above strongly suggest, but do not directly prove, that each of the five acid-eluted peptides are also successfully translocated by TAP and bind to MHC class I. To directly address affinity for TAP, binding assays were performed using microsomes isolated from insect cells expressing human TAP1–TAP2 complexes (28). Relative affinities (IC₅₀ values) measurable in this assay range from 0.1 to 3,000. Peptides with IC₅₀ values >3,000 are excluded from translocation into the endoplasmatic reticulum (ER) (43). The IC₅₀ values suggest a slightly higher TAP-binding affinity for the two HLA-A2 ligands than for the three
30-mer Nef\textsubscript{123–152}. The sequence of the peptides contained in the major chromatogram obtained upon separation of the proteasomal digest of the polypeptide HIV-1 Nef\textsubscript{123–152}. A synthetic peptide corresponding to the sequence Nef\textsubscript{123–152} was incubated with proteasomes isolated from human T1 cells (see Materials and Methods). The peptide mixture obtained after substrate consumption was separated by rp-HPLC, and individual fractions were analyzed by mass spectrometry and Edman degradation. (A) rp-HPLC chromatogram obtained upon separation of the proteasomal digest of the 30-mer Nef\textsubscript{123–152}. The sequence of the peptides contained in the major peaks is indicated in single-letter code. The Nef HLA-A2–binding ligands, 126PLTFGWCYKLV\textsubscript{146}, as well as the Nef HLA-B7–binding ligands, 128TPGPGVRY\textsubscript{135} and 128TPGPGVRYPL\textsubscript{137}, and 136PLTFGWCYKL\textsubscript{145} and 136PLTFGWCYKLV\textsubscript{146}, as well as the Nef HLA-A2–binding ligands, 126PLTFGWCYKLV\textsubscript{146}, are indicated by asterisks. (B) Digestion map compiling all identified degradation products (indicated as lines underneath the sequence; the five MHC class I ligands are shown in bold) and proteasomal cleavage sites (indicated by arrows). The strength of proteasomal cleavage was estimated from quantification of the adjacent products by Edman degradation. The six arrow sizes indicate increasing amounts of peptides flanking each cleavage site as follows: undetectable by Edman degradation; 10, 10–50, 50–150, 150–500, and 500 pmol peptide.

Figure 4. Digestion by 20S proteasomes of the synthetic 30-mer polypeptide HIV-1 Nef\textsubscript{123–152}. A synthetic peptide corresponding to the sequence Nef\textsubscript{123–152} was incubated with proteasomes isolated from human T1 cells (see Materials and Methods). The peptide mixture obtained after substrate consumption was separated by rp-HPLC, and individual fractions were analyzed by mass spectrometry and Edman degradation. (A) rp-HPLC chromatogram obtained upon separation of the proteasomal digest of the 30-mer Nef\textsubscript{123–152}. The sequence of the peptides contained in the major peaks is indicated in single-letter code. The Nef HLA-A2–binding ligands, 126PLTFGWCYKLV\textsubscript{146}, as well as the Nef HLA-B7–binding ligands, 128TPGPGVRY\textsubscript{135} and 128TPGPGVRYPL\textsubscript{137}, and 136PLTFGWCYKL\textsubscript{145} and 136PLTFGWCYKLV\textsubscript{146}, as well as the Nef HLA-A2–binding ligands, 126PLTFGWCYKLV\textsubscript{146}, are indicated by asterisks. (B) Digestion map compiling all identified degradation products (indicated as lines underneath the sequence; the five MHC class I ligands are shown in bold) and proteasomal cleavage sites (indicated by arrows). The strength of proteasomal cleavage was estimated from quantification of the adjacent products by Edman degradation. The six arrow sizes indicate increasing amounts of peptides flanking each cleavage site as follows: undetectable by Edman degradation; 10, 10–50, 50–150, 150–500, and 500 pmol peptide.

**Discussion**

Although it is undisputed that the COOH termini of proteasome-dependent epitopes are predominantly generated by proteasomal cleavage, it is controversial whether proteasomes also contribute significantly to liberation of the NH\textsubscript{2} termini of MHC class I ligands (1). Here we describe five naturally processed MHC class I binding peptides of HIV-1 N ef, corresponding to three different CTL epitopes. These are the first naturally processed peptides ever identified in HIV N ef. In contrast to previous reports on this subject, our study concerns not only a single but also a cohort of determinants derived, in addition, from a highly relevant antigen. Four results in combination strongly suggest the generation of these determinants by proteasomes without assistance of other proteases: (a) all five ligands studied were produced in definitive form by proteasomes in digests of the fragment HIV-1 N ef\textsubscript{123–152} (this paper) as well as of recombinant full-length N ef (Lucchiari-Hartz, M., N. Hitziger, K. Eichmann, and G. Niedermann, manuscript in preparation); (b) the NH\textsubscript{2} termini of four of the five ligands correspond to major proteasomal cleavage sites; (c) Nt-extended proteolytic fragments were found in proteasomal digests for only one of the five definitive ligands; and (d) all five peptides were transported by TAP.

H LA-B7 ligands (Table II). Of note, all five HLA-binding peptides have a proline in either position 1 or 2, a situation thought to be unfavorable for TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC\textsubscript{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45).
Unexpectedly, four of the five ligands represent COOH-terminal length variants of only one HLA-B7- (8- and 10-mer) and one HLA-A2-binding epitope (10- and 11-mer), respectively. To our knowledge, this is the first description of length variants of CTL epitopes from a non-self-antigen. However, since length variants were seen for two out of three CTL epitopes, this observation might not be exceptional. In both cases, CTL lines induced with the short and the long variant peptide showed CTL cross-recognition of both peptide length variants. However, since we did not analyze CTLs on the clonal level, it cannot be excluded that CTLs with exclusive specificity for the inducing peptide are also induced. The length variants described here are COOH-terminal length variants. Thus, our finding is in agreement with the notion that there is apparently no COOH-terminal trimming activity in the ER (46, 47), and also no evidence for effective COOH-terminal trimming activity in the cytosol (12). Recognition of the longer variants by CTLs was not dependent on extracellular trimming by carboxypeptidases present in FCS (data not shown). Two binding modes for class I ligands slightly longer than canonical peptides have been described: they either pro-duce beyond the COOH-terminal end of the MHC class I peptide binding groove (48) or are fixed at the COOH-terminus and bulge out in the middle (49, 50). Since we observe extensive CTL cross-recognition, we favor the former binding mode for the longer epitope variants in both of our pairs of class I ligands as being more likely to be compatible with conserved conformation of the central peptide residues critical for TCR recognition.

In four of the five ligands, the NH2 termini coincide with major proteasome cleavage sites. In the case of all five ligands, not only the COOH-terminal aa, but also the aa in the flanking position N minus 1, are preferred P1 residues2 of proteasomes (either tyrosine, leucine, valine, or arginine). Four ligands have small or polar aa in the NH2-terminal position of proteasomal cleavage sites. The major cleavage site that creates the COOH terminus of the abundant octamer HLA-B7–binding epitope and the NH2 terminus of the two HLA-A2 ligands has a valine in position P3. Small and/or polar P1 residues and hydrophobic P3 residues can promote proteasomal cleavage at the P1–P1' position of proteasomal cleavage sites. The major cleavage site that creates the COOH terminus of the abundant octamer HLA-B7–binding epitope and the NH2 terminus of the two HLA-A2 ligands has a valine in position P3. Small and/or polar P1 residues and hydrophobic P3 residues can promote proteasomal cleavage at the P1–P1' position of proteasomal cleavage sites. The major cleavage site that creates the COOH terminus of the abundant octamer HLA-B7–binding epitope and the NH2 terminus of the two HLA-A2 ligands has a valine in position P3. Small and/or polar P1 residues and hydrophobic P3 residues can promote proteasomal cleavage at the P1–P1' position of proteasomal cleavage sites. The major cleavage site that creates the COOH terminus of the abundant octamer HLA-B7–binding epitope and the NH2 terminus of the two HLA-A2 ligands has a valine in position P3. Small and/or polar P1 residues and hydrophobic P3 residues can promote proteasomal cleavage at the P1–P1' position of proteasomal cleavage sites. The major cleavage site that creates the COOH terminus of the abundant octamer HLA-B7–binding epitope and the NH2 terminus of the two HLA-A2 ligands has a valine in position P3. Small and/or polar P1 residues and hydrophobic P3 residues can promote proteasomal cleavage at the P1–P1' position of proteasomal cleavage sites.

Four HLA ligands had low to moderate affinities for human TAP, whereas the 130PTFTGWCYKL146 HLA-A2 ligand had a slightly higher TAP affinity. All five ligands have proline in either position 1 or 2. It has been recognized previously that prolyl residues in positions 1, 2, or 3 are generally unfavorable for TAP translocation (28, 44, 45, 52). In such cases, it has been proposed that NH2-terminal

\[\text{TAP}^*\]

\[\text{MHC class I binding}\]

\[\text{No. of MHC I ligands per cell}^a\]

**Table II.** Characteristics of HIV-1 Nef CTL Epitopes Described in This Study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Relative IC50 for TAP</th>
<th>MHC class I binding</th>
<th>No. of MHC I ligands per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nef136–145</td>
<td>PLTFGW CYKL</td>
<td>49</td>
<td>295</td>
<td>85</td>
</tr>
<tr>
<td>Nef136–146</td>
<td>PLTFGW CYKLV</td>
<td>17</td>
<td>75</td>
<td>125</td>
</tr>
<tr>
<td>HLA-B7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nef128–135</td>
<td>TPGPVGY</td>
<td>160</td>
<td>30</td>
<td>3,600</td>
</tr>
<tr>
<td>Nef128–137</td>
<td>TPGPVGYPL</td>
<td>195</td>
<td>25</td>
<td>840</td>
</tr>
<tr>
<td>Nef135–143</td>
<td>YPLTFGW CY</td>
<td>304</td>
<td>18</td>
<td>80</td>
</tr>
</tbody>
</table>

*The unlabeled Nef peptides were used to compete the binding of 125I-labeled RRRYASTEL (used at 240 nM) to TAP1/2 insect cell microsomes. The average IC50 of the reporter peptide in these assays was 400 nM. All values are normalized by dividing IC50 for test peptides by the IC50 of unlabeled reporter peptide measured in the same assay.

†The number of Nef ligands per Nef T1 cell (HLA-A2 restriction) and Nef Jurkat cell (HLA-B7 restriction) was estimated as described in Materials and Methods (see also Fig. 5). Numbers given are corrected for extraction yields. Extraction yields: Nef136–145 (69%), Nef136–146 (74%), Nef128–135 (88%), Nef128–137 (95%), and Nef135–143 (50%).

‡The unlabeled Nef peptides were used to compete the binding of 125I-HBV core 18–27 (6Y) to HLA-A2 molecules. The average IC50 of the reporter peptide in these assays was 80 nM. All values are normalized by dividing IC50 of unlabeled reporter peptide measured in the same assay.

§The unlabeled Nef peptides were used to compete the binding of 125I-labeled RRYNASTEL (used at 240 nM) to TAP1/2 insect cell microsomes. The average IC50 of the reporter peptide in these assays was 400 nM. All values are normalized by dividing IC50 for test peptides by the IC50 of unlabeled reporter peptide measured in the same assay.

A low fluorescence index (FR) for Nef198LHPEYFKNC206 (negative control) = 8, for the HLA-B7–restricted self-peptide APRTVALTAL (positive control) = 45.
extension can improve TAP affinity, and that the final class I ligands may be produced by trimming of epitope precursors in the ER (52, 53). However, we have recently presented evidence suggesting that even for presentation by HLA-A2, which is suboptimally adapted to TAP, antigen processing may favor peptides that do not require ER processing (43, 45). For the two HLA-A2 ligands and for two of the three HLA-B7 ligands identified in this study, Nt-extended precursor peptides were not found among the proteasomal products. One of the potential precursors of the HLA-B7-binding octamer Nef128TPGPGVRY135 was the nonamer YTPGPGVRY. However, this nonamer is generated in lower amounts than the octamer. In addition, it has a proline in position 3 and should therefore not be preferred in TAP transport. The 10-mer NYTPGPGVRY and the 12-mer WQNYTPGPGVRY might have higher TAP affinity because they lack a prolyl residue in positions 1–3. Although these peptides are generated in significantly smaller amounts than the octamer, a contribution of NH2-terminal trimming of these putative precursors in the ER is presently not excluded and needs to be evaluated. The only proteasomal fragment that could represent an Nt-elongated precursor of the HLA-B7–binding 10-mer ligand NefTPGPGVRYPL137 was a single cleavage product starting with the NH2-terminus of the 30-mer substrate. A similar single cleavage intermediate was found in significant amounts for the HLA-B7–binding octamer. However, these single cleavage intermediates were not found in digests of full-length Nef (Lucchiari-Hartz, M . N. Hitziger, K. Eichmann, and G. Niedermann, manuscript in preparation) and most probably result from the limited length of the substrate used here. Together, our data suggest that at least four of the five HLA-A2 and HLA-B7 ligands identified here are translocated into the ER predominantly in their definitive form, despite suboptimal TAP transport. We have shown previously that peptides with similar TAP affinities can even be very efficiently presented when abundantly generated (43). Of note, it has been shown that even the highly restrictive mouse TAP translocates peptides with unfavorable COOH-terminal residues in amounts sufficient for T cell recognition. The selective TAP influence became detectable only at limiting cytosolic peptide concentrations (54).

Several previous studies showing that MHC class I ligands may be direct major products of 20S proteasomes and/or proteasome–PA28 complexes were concerned with highly selected examples, i.e., the high copy self-peptides SYFPEITHI and TLWVDPYEV, derived from the cellular tyrosine kinase Janus kinase (JAK) 1, and the product of the B cell translocation gene 1, respectively (4, 8, 55), or the immunodominant OVA epitope SIINFEKL (3, 4, 56). Slightly longer precursor peptides that could be candidates for NH2-terminal trimming were either not found or were produced in low quantities in these cases. A second group of MHC ligands was shown to be generated by proteasomes as minor products. The Ld ligand YPHFMPTNL derived from the pp89 protein of the murine cytomegalovirus and the subdominant OVA epitope KVVRFDKL are produced by proteasomes, albeit in small amounts, whereas Nt-extended epitopes are more efficiently produced (3, 8, 57, 58). A p53-derived and a β-galactosidase–derived class I ligand could also be detected in proteasomal digests of a source polypeptide and the source protein, respectively, albeit only with the highly sensitive use of specific CTLs (27, 56). Three further reports describe unsuccessful attempts to detect epitopes in proteasomal digests: the KSPWFITTL peptide derived from the p89 protein of the murine cytomegalovirus and the subdominant OVA epitope KVVRFDKL are produced by proteasomes, albeit in small amounts, whereas Nt-extended peptides are more efficiently produced (3, 8, 57, 58). A p53-derived and a β-galactosidase–derived class I ligand could also be detected in proteasomal digests of a source polypeptide and the source protein, respectively, albeit only with the highly sensitive use of specific CTLs (27, 56). Three further reports describe unsuccessful attempts to detect epitopes in proteasomal digests: the KSPWFITTL peptide derived from the p89 protein of the murine cytomegalovirus and the subdominant OVA epitope KVVRFDKL are produced by proteasomes, albeit in small amounts, whereas Nt-extended peptides are more efficiently produced (3, 8, 57, 58). A p53-derived and a β-galactosidase–derived class I ligand could also be detected in proteasomal digests of a source polypeptide and the source protein, respectively, albeit only with the highly sensitive use of specific CTLs (27, 56). Three further reports describe unsuccessful attempts to detect epitopes in proteasomal digests: the KSPWFITTL peptide derived from the p89 protein of the murine cytomegalovirus and the subdominant OVA epitope KVVRFDKL are produced by proteasomes, albeit in small amounts, whereas Nt-extended peptides are more efficiently produced (3, 8, 57, 58). A p53-derived and a β-galactosidase–derived class I ligand could also be detected in proteasomal digests of a source polypeptide and the source protein, respectively, albeit only with the highly sensitive use of specific CTLs (27, 56).
(60). In these cases, only slightly longer peptides were found by protein analytical methods. However, in one of these studies, minor products have not been analyzed (60). Furthermore, the exact NH$_2$-terminus of human melanoma antigen (MAGE)3$_{172-179}$ is liberated by proteasomes. Nevertheless, the epitope is cryptic, because its COOH terminus is normally not liberated by proteasomes. However, crypticity of this epitope is abolished in the presence of lactacystin, since lactacystin-treated proteasomes generate the epitope COOH terminus in addition to its NH$_2$ terminus (61). Together, these data suggest that proteasomes often produce the final MHC class I ligands, albeit in varying amounts. Therefore, the available data have not led to a general consensus on the role of proteasomes in antigen processing.

The notion that epitope NH$_2$ termini may frequently result from nonproteasomal cleavage in vivo stems primarily from a study on the generation of SIINFEKL in cells transfected with minigenes. Although production of the epitope from Ct-extended versions was inhibited by 2 or 20 μM lactacystin, that from Nt-extended versions was not (11). This finding may have an alternative explanation. The peptide bond at the SIINFEKL NH$_2$ terminus is hydrolyzed efficiently by purified 20S proteasomes and proteasome-PA28 complexes (3, 4, 56). Since the SIINFEKL NH$_2$ terminus is directly preceded by glutamic acid (E), cleavage of the E$_{256}$-S$_{257}$ bond is most likely dependent on the post-glutamyl activity of the proteasome. This activity is only marginally and competitively inhibited by lactacystin, i.e., only partially even at excessive lactacystin concentrations (62). Thus, it is to be expected that proteasomal generation of the SIINFEKL NH$_2$ terminus is poorly inhibited by lactacystin. Moreover, it is unlikely that the extraordinarily rapid hydrolysis by proteasomes at this site is efficiently blocked by any competitive inhibitor.

Along the same line, it was proposed that the SIINFEKL NH$_2$ terminus is produced by LAP, a cytosolic aminopeptidase inducible by IFN-γ. Small amounts of SIINFEKL were shown to be produced upon prolonged incubation of synthetic QLESIINFEKL with cytosol preparations from IFN-γ-treated cells, or by purified LAP (12). We think that this study potentially overemphasizes a minor mechanism in the generation of SIINFEKL. This epitope is excised by purified proteasomes from partial or total OVA in 7-10-fold greater amounts than QLESIINFEKL (3, 56). In addition, SIINFEKL is resistant against proteasomal attack once generated (3). In contrast, SIINFEKL is degraded when incubated with LAP (our unpublished data). Thus, the production of SIINFEKL via QLESIINFEKL by a two-step digestion involving aminopeptidases is likely to be a minor pathway. In addition, TPP II, which was inadvertently depleted from the cytosol in the protocol used by Beninga et al. (12), is a strong candidate for the generation of SIINFEKL via the QLESIINFEKL precursor.

We do not mean to exclude that precursor trimming (at least NH$_2$-terminal trimming) also contributes to MHC class I ligand formation. The steric constraints on peptide extensions at the NH$_2$ terminus of the class I peptide binding groove appear to be stricter than on peptide extensions at the COOH terminus (63). In accordance with that fact, alignments of eluted class I ligands (6) and MHC-peptide affinity measurements (64) suggest that stable complex formation between class I MHC molecules and Nt-extended peptides may not occur normally, although there may be exceptions (65). These stringent requirements for correct NH$_2$ termini in MHC class I ligands are compatible with a trimming activity generating suitable termini. This activity may act in cases where proteasomes do not generate the correct NH$_2$ terminus of a class I ligand. Moreover, trimming may often contribute to ligand formation when elongated peptides are produced by proteasomes in addition to the minimal ligands, especially when such precursors have significantly higher TAP affinities than the minimal epitopes. Indeed, experimental evidence for NH$_2$-terminal trimming capacity has been reported in the cytosol (12) and in the ER for both signal peptide–coupled epitope precursors (11, 66-68) and a TAP-translocated peptide (43). However, it remains to be determined whether the generation of an epitope by two distinct proteases is as efficient as the generation of an epitope by proteasomes alone.

Nef is a major virulence factor of HIV and simian immunodeficiency virus (SIV), and appears to be critical for the development of AIDS (69-71). Among other biological effects, Nef downregulates MHC class I expression in HIV-infected and Nef-transfected cells (72), and was suggested to partially protect HIV-infected primary CD4$^+$ T cells against recognition by HLA-A2–restricted HIV-Gag and HIV reverse transcriptases–specific CTL clones (73). On the other hand, there is also strong evidence that CD8$^+$ lymphocytes play an important role in controlling viremia in SIV and HIV infections (74, 75). An effective HIV vaccine should therefore be designed to elicit CTL responses. Before the present report, only two naturally processed CTL epitopes of HIV were known, one for Gag and the other for reverse transcriptase (26). In view of the MHC downregulation by Nef, it may be mandatory to target CTL vaccines to epitopes that are presented in high copy numbers, such as the HLA-B7–restricted naturally processed Nef peptide, $\text{V}_{128}^\text{PGPVR}_{135}$, identified in this paper. Nef is synthesized at the earliest stage of viral gene expression and is abundantly expressed (76). High anti-Nef CTL responses have been detected in the acute and asymptomatic phases as well as later in HIV infection (35, 77, 78), and high frequencies of CTL precursors have been found in noninfected individuals (20). A detailed knowledge of MHC class I–peptide ligands and their intracellular generation, as well as the molecular basis of the adverse effects of Nef, should be instrumental in the development of an HIV vaccine including Nef CTL epitopes.

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