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 NH_2 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 NH_2 termini correspond to major proteasome cleavage sites, and putative NH_2-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 NH_2-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 NH_2 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 NH_2 termini of proteaso-

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[1]*Abbreviations used in this paper:* aa, amino acid(s); ER, endoplasmatic reticulum; FR, fluorescence ratio; IC_{50}, 50% inhibition of specific binding; LAP, leucine aminopeptidase; Nt, NH_2-terminally; PA28, proteasome activator 28; rp-HPLC, reversed phase HPLC; TAP, transporter associated with antigen processing; TPP II, tripeptidyl peptidase II.

mal 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 20S or 26S 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 processive, 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 NH_2 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 NH_2-terminally (Nt)-extended versions was not. Moreover, SIINFEKL could be produced from the Nt-extended fragment QLESIINFEKL by leucine aminopeptidase (LAP), a cytosolic peptidase inducible by IFN-γ (12). This was put forward as evidence that the SIINFEKL NH_2 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 NH_2 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, $Nef_{123–152}$, 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 ligands 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 NH_2 123–152 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 pSBBRU6e containing the sequence encoding the whole Nef protein from HIV-1 strain LAI, under the control of the human metallothionein IIA 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 ^{51}Cr 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 5×10^6) were incubated in 24-well culture plates (Nalge Nunc International) using 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 μg/ml 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 1×10^6 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 1×10^5 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 ^{51}Cr-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

(rp-HPLC) fractions containing naturally processed peptides or proteasomal products. To study the effect of proteasome inhibitors, the Nef-expressing cells were incubated in the presence of 10 μM lactacystin (Dr. E.J. Corey, Harvard Medical School, Boston, MA) or 100 μM *N*-acetyl-leucinyl-leucinyl-norleucinal (LLnL; Sigma Chemical Co.) for 2 h before ^{51}Cr labeling. The cells were then radiolabeled, and the preexisting peptide–MHC class I complexes on the cell surface were removed by exposure to a quick acid wash (131 mM citric acid, 66 mM disodium phosphate, pH 3.1) at 25°C for 3 min (24). After neutralization in 30 vol of complete RPMI 1640 medium and washing in PBS, cells were plated and used as targets in a 4-h ^{51}Cr-release assay in either the presence or absence of the inhibitors.

Synthetic Peptides. Peptides were either synthesized using solid phase 9-fluorenylmethoxycarbonyl (F-moc) chemistry on an Applied Biosystems 431A peptide synthesizer or purchased from Genosys Biotechnologies. Peptides were purified to >98% homogeneity by rp-HPLC. 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 10^9 Nef$^-$ T1, 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 rp-HPLC.

In Vitro Digestion of Polypeptide 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. Tripeptidyl peptidase II (TPP II) contaminations were excluded by use of the TPP II inhibitors AAF-chloromethylketone (Bachem) and PMSF (Sigma Chemical Co.). Digestion of the synthetic 30-mer polypeptide 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 $MgCl_2$, 0.5 mM 2-ME, and 0.02% azide. Aliquots of the reaction mixture were fractionated by rp-HPLC.

rp-HPLC Fractionation and Analysis of Natural and Synthetic Peptides. Aliquots of rp-HPLC fractions were separated by rp-HPLC (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 was removed from the eluted fractions by lyophilization. The lyophilized material was redissolved in 200 μl PBS and stored at −70°C. Aliquots of the rp-HPLC fractions were assayed for recognition by specific CTLs in a ^{51}Cr-release assay. Aliquots of the rp-HPLC fractions from proteasomal digestions were directly analyzed with a G2025A matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Hewlett-Packard) and by Edman degradation on a Hewlett-Packard instrument (model G1000A).

The Transporter Associated with Antigen Processing–Peptide Binding Assay. The affinity of peptides for the transporter associated with antigen processing (TAP) was measured essentially as described previously (28), using microsomes purified from Sf9 insect cells expressing human TAP1–TAP2 complexes, iodinated reporter peptide RRYNASTEL, and a dilution series of competitor 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.

MHC Class I–Peptide Binding Assay. The HLA binding ability of the HLA-B7–restricted peptides was measured by HLA-B7 stabilization on the TAP-deficient hybrid cell line T2-B7 (29). In brief, cells were incubated with 100, 50, 25, and 12.5 μM of peptide in serum-free medium at 37°C. Staining for indirect immunofluorescence was performed with ME1 (anti-B7, -Bw22, and -B27) as first antibody, and FITC-labeled anti–mouse IgG (Sigma Chemical Co.) as second antibody. Fluorescence intensities were measured on a FACScan™ cytometer (Becton Dickinson). The fluorescence 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$_{198}$LHPEYFKNC$_{206}$, which does not bind to HLA-B7, was used as negative control.

Peptide affinity for immunoaffinity-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 Jesthorn using Sepharose-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 unlabeled competitor peptides in a total volume of 30 μl PBS buffer with 0.05% NP-40 and 1 mM PMSF. 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 MHC Class I Ligands in Nef-transfected Cells. The molar concentrations of MHC ligands in rp-HPLC fractions of acid eluates of Nef-transfected cells were determined by titration of rp-HPLC fractions in a 4-h ^{51}Cr-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 Avogadro'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 protease complex TPP II (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 TPP II. Nef$^+$ C1R-A2 and Nef$^+$ C1R-B7 cells were incu-

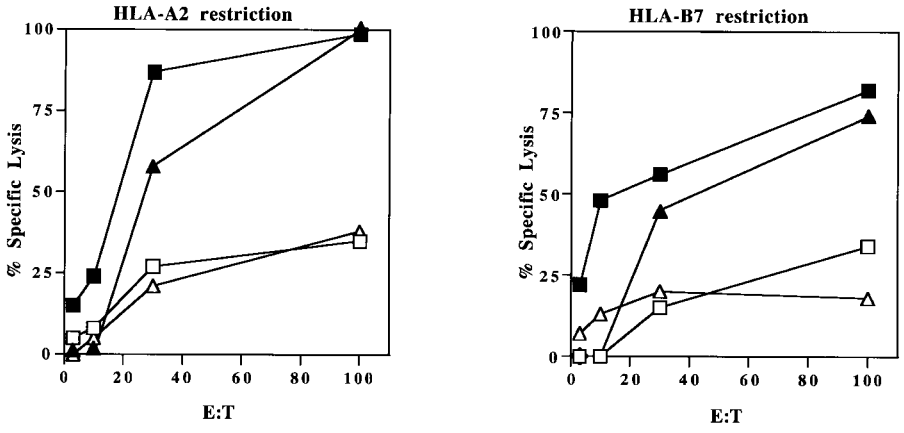

Figure 1. Effect of lactacystin on recognition of HLA-A2– and HLA-B7–restricted HIV-1 Nef-derived epitopes by Nef-specific CTL lines. ^{51}Cr-labeled C1R-A2 (left) and C1R-B7 (right) cells, untransfected (Nef$^-$) or transfected (Nef$^+$), were preincubated for 2 h in the presence of 10 μM lactacystin. Cell surface MHC class I–peptide complexes were then removed by acid treatment (see Materials and Methods). The cells were washed and used as target cells in a 4-h ^{51}Cr-release assay in the absence (■) or presence of 10 μM lactacystin (△). The Nef-specific CTL line was generated from PBLs of an uninfected seronegative donor after in vitro stimulation with Nef$^+$ C1R-A2 or Nef$^+$ C1R-B7, as described in Materials and Methods. Reconstitution of lactacystin-treated Nef$^+$ C1R-A2 cells was achieved by pulsing with the A2-restricted HIV-1 Nef$_{136}$PLTFGWCYKL$_{145}$ peptide and reconstitution of Nef$^+$ C1R-B7 cells by pulsing with the B7-restricted HIV-1 Nef$_{128}$TPGPGVRYPL$_{137}$ peptide (▲). Nef$^-$ C1R-A2 and Nef$^-$ C1R-B7 cell lines were included as negative controls (□). The results are representative of three independent 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 ^{51}Cr-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 (Nef$_{123–152}$, Table I) was chosen for this study. One HLA-A2 (Nef$_{136}$PLTFGWCYKL$_{145}$) and two HLA-B7 (Nef$_{128}$TPGPGVRYPL$_{137}$ and Nef$_{135}$YPLTFGWCY$_{143}$) 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

Table I. *CTL Epitopes Previously Predicted by Epitope Mapping with Synthetic Peptides and/or Peptide–HLA Binding Assays in the 123–152 Region of Nef of HIV-1 LAI*

Position	Sequence*	HLA class	Reference
126–138	NYTPGPGVRYPLT	B7	33
128–137	TPGPGVRYPL	B7	34, 35
130–143	GPGVRYPLTFGWCY	B57	36
132–147	GVRYPLTFGWCYKLVP	A1, B8, B18, B49	37, 38
133–148	VRYPLTFGWCYKLVPV	B57	39
134–143	RYPLTFGWCY	B18, B49	37
134–144	RYPLTFGWCYK	B18, B49	37, 40
135–143	YPLTFGWCY	B7, B18, B49	37
135–144	YPLTFGWCYK	B18, B49	37
136–145	PLTFGWCYKL	A2.1	35

*The single-letter amino acid code is used.

rarely been successful (41, 42). To overcome these problems, we used a regulated Nef expression vector (pSBBRUnef) 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 pSBBRUnef 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 $Nef_{123–152}$ 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 $Nef_{123–152}$, 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

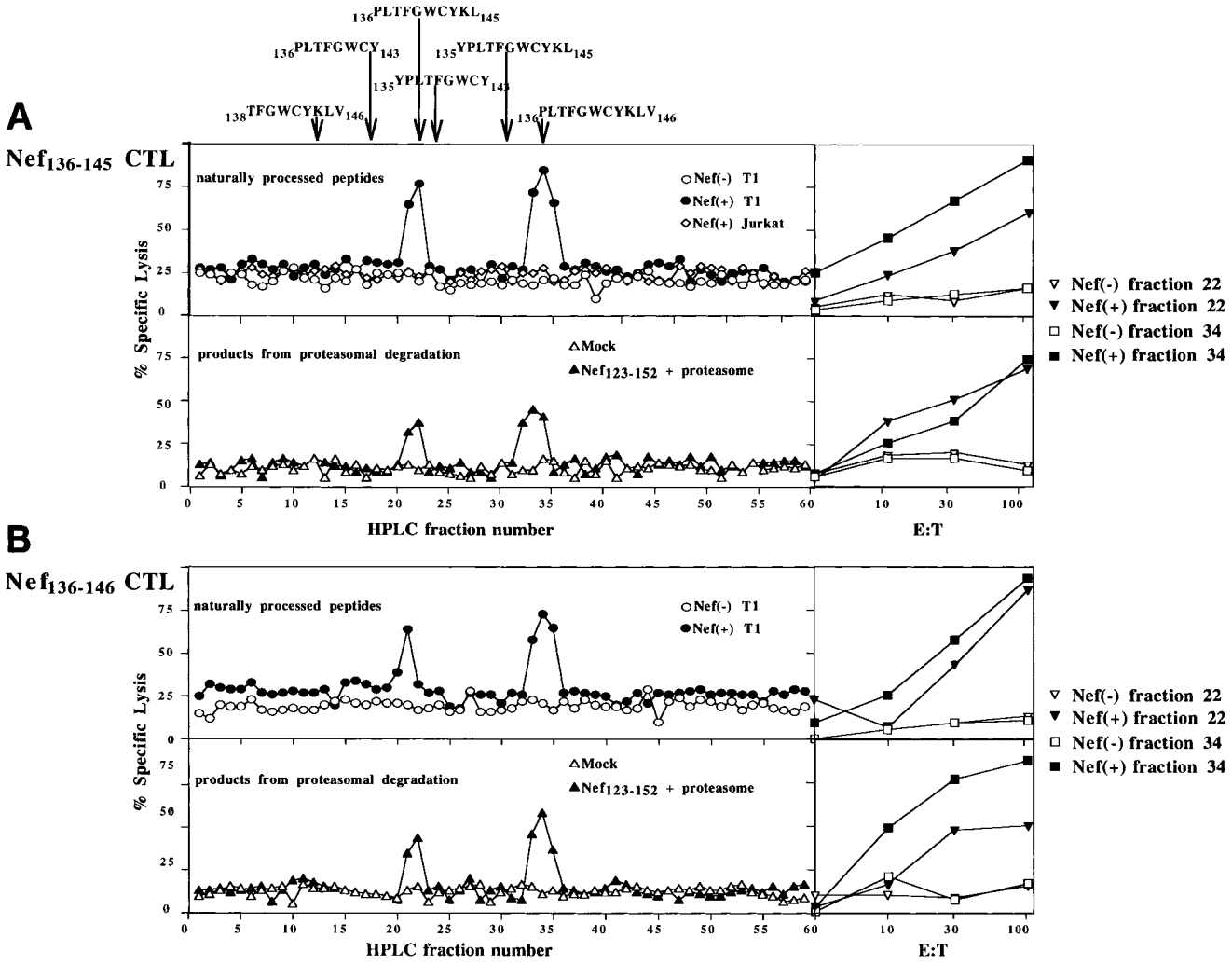

Figure 2. Comparison between HLA-A2–restricted naturally processed HIV-1 Nef peptides and products derived from in vitro 20S proteasomal degradation of the synthetic polypeptide HIV-1 $Nef_{123–152}$. Acid-soluble extracts prepared from Nef^+ T1 cells and peptide products derived from 20S proteasome–mediated degradation of the synthetic 30-mer polypeptide $Nef_{123–152}$ 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 $Nef_{136}PLTFGWCYKL_{145}$ (A) and $Nef_{136}PLTFGWCYKLV_{146}$ (B) in a 4-h ^{51}Cr-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 $Nef_{136}PLTFGWCYKL_{145}$ peptide–specific CTL line did not lyse P815-A2 cells pulsed with peptides eluted from HLA-$A2^-$, Nef^+ 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.

not transfected with Nef and from Nef-transfected cells lacking the MHC molecule in question were fractionated using the same gradient and tested by the same CTLs.

Comparison between HLA-A2–restricted Naturally Processed Nef Peptides and Peptides Derived from Proteasomal Degradation of the 30-mer $Nef_{123–152}$ 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}PLTFGWCYKL$_{145}$ (Fig. 2 A, top). Surprisingly, Nef_{136}PLTFGWCYKL$_{145}$-specific CTLs recognized not only fractions coeluting with the synthetic peptide Nef_{136}PLTFGWCYKL$_{145}$ (fraction 21/22), but also fractions coeluting with the synthetic peptide Nef_{136}PLTFGWCYKLV$_{146}$ (fractions 33–35). Accordingly, a CTL line was generated by stimulation with Nef_{136}PLTFGWCYKLV$_{146}$. As shown in Fig. 2 B (top), these CTLs also recognized fractions containing Nef_{136}PLTFGWCYKL$_{145}$, as well as fractions containing Nef_{136}PLTFGWCYKLV$_{146}$. 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}PLTFGWCYKLV$_{146}$-specific CTLs not shown). These controls indicate that the acid-eluted peptides recognized by both CTL lines were 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 $Nef_{123–152}$ 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}TPGPGVRYPL$_{137}$. Again surprisingly, Nef_{128}TPGPGVRYPL$_{137}$-specific CTLs recognized two rp-HPLC fractions of acid-eluted material: fraction 3 corresponding to the elution time of Nef_{128}TPGPGVRY$_{135}$, and fraction 15 corresponding to the elution time of Nef_{128}TPGPGVRYPL$_{137}$ (Fig. 3 A, top). Accordingly, CTLs were prepared against the smaller peptide, the octamer Nef_{128}TPGPGVRY$_{135}$, 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_{135}YPLTFGWCY$_{143}$. 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_{135}YPLTFGWCY$_{143}$ 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}PLTFGWCYKL$_{145}$ and Nef_{136}PLTFGWCYKLV$_{146}$ 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}TPGPGVRY$_{135}$ 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}TPGPGVRYPL$_{137}$ and Nef_{135}YPLTFGWCY$_{143}$ 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 NH_2 termini of the two naturally processed HLA-A2 ligands. Most signifi-

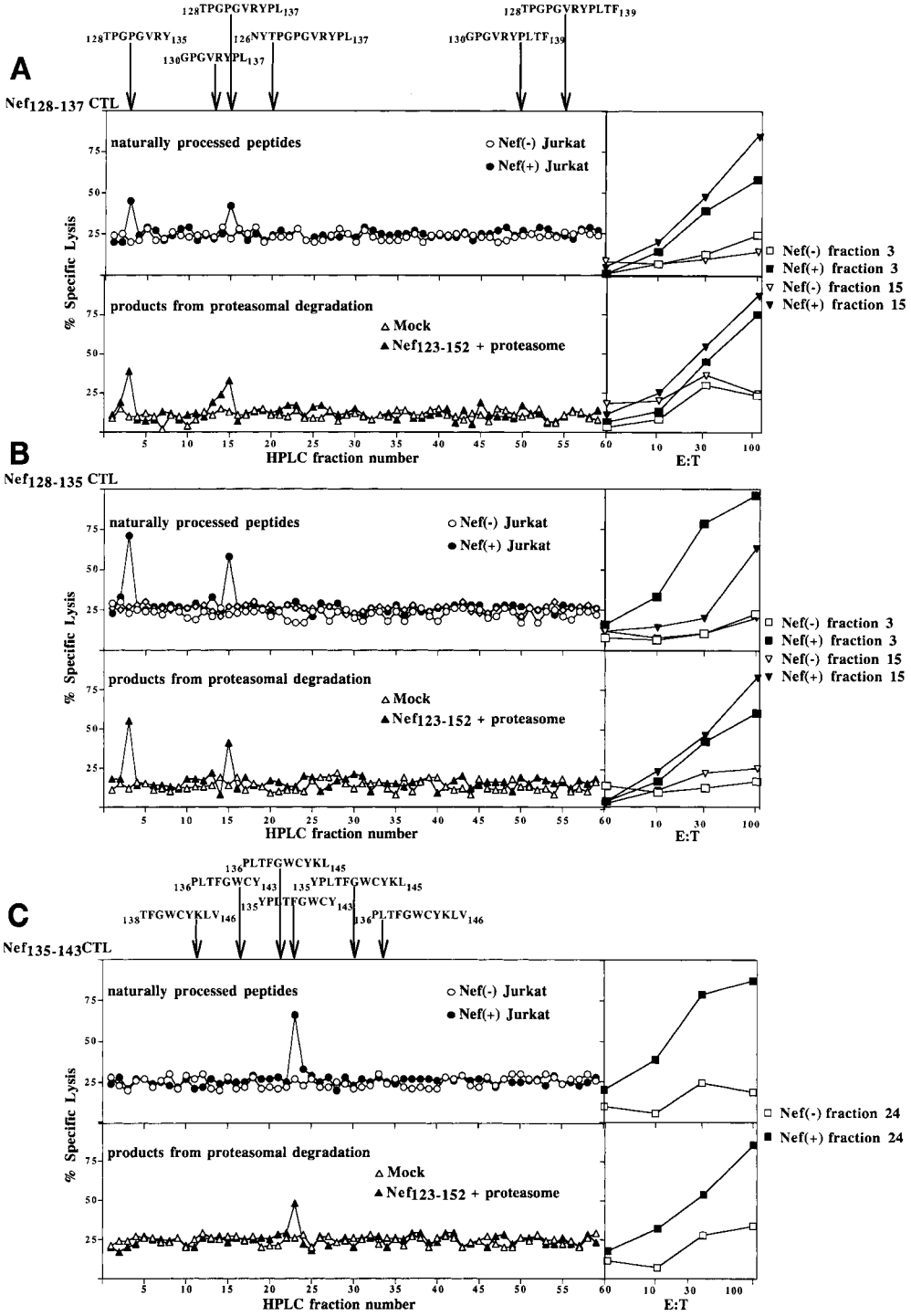

Figure 3. Comparison between HLA-B7–restricted naturally processed HIV-1 Nef peptides and products derived from in vitro 20S proteasomal degradation of the synthetic polypeptide HIV-1 $Nef_{123–152}$. Acid-soluble extracts prepared from Nef^+ Jurkat cells and peptide products derived from 20S proteasome–mediated degradation of the synthetic 30-mer polypeptide $Nef_{123–152}$ 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-B7 cells for lysis by CTL lines specific for peptides $Nef_{128}TPGPGVRYPL_{137}$ (A), $Nef_{128}TPGPGVRY_{135}$ (B), or $Nef_{135}YPLTFGWCY_{143}$ (C) in a 4-h ^{51}Cr-release assay. Acid-soluble extracts from Nef^- Jurkat cells fractionated before the eluate of Nef^+ Jurkat cells (A, B, and C, top left), or mock rp-HPLC fractions (buffer only) collected before fractionation of the proteasomal products (A, B, and C, bottom left), gave no activity. The B7-restricted $Nef_{128}TPGPGVRY_{135}$ peptide–specific CTL lines did not lyse P815-B7 cells pulsed with peptides eluted from HLA-$B7^-$, Nef^+ T1 cells (B, top left). CTL assays were carried out using an E/T ratio of 50:1 (A, B, and C, left) or at different E/T ratios as indicated (A, B, and C, right). The elution position of synthetic peptides is indicated by arrows. The results are representative of five independent experiments.

cantly, in four of the five naturally processed peptides ($Nef_{128}TPGPGVRY_{135}$, $_{128}TPGPGVRYPL_{137}$, $_{136}PLTFGWCYKL_{145}$, and $_{136}PLTFGWCYKLV_{146}$), the NH_2 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 (IC_{50} values) measurable in this assay range from 0.1 to 3,000. Peptides with IC_{50} values >3,000 are excluded from translocation into the endoplasmatic reticulum (ER [43]). The IC_{50} values suggest a slightly higher TAP-binding affinity for the two HLA-A2 ligands than for the three

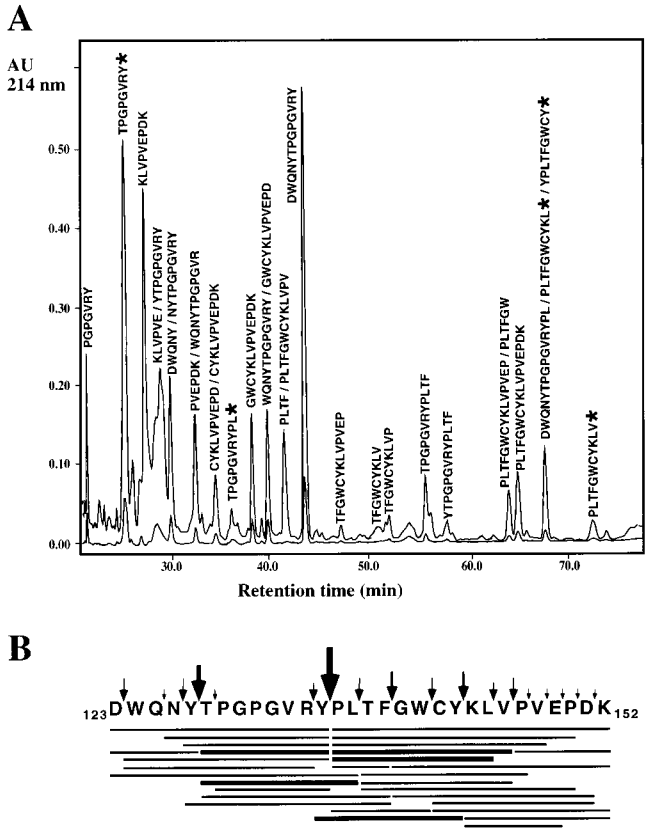

Figure 4. Digestion by 20S proteasomes of the synthetic 30-mer polypeptide HIV-1 $Nef_{123–152}$. A synthetic peptide corresponding to the sequence $Nef_{123–152}$ was incubated with proteasomes isolated from human T1 cells (see Materials and Methods). The peptide mixture obtained after substrate consumption 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 $Nef_{123–152}$. The sequence of the peptides contained in the major peaks is indicated in single-letter code. The Nef HLA-A2–binding ligands, $_{136}$PLTFGWCYKL$_{145}$ and $_{136}$PLTFGWCYKLV$_{146}$, as well as the Nef HLA-B7–binding ligands, $_{128}$TPGPGVRY$_{135}$, $_{128}$TPGPGVRYPL$_{137}$, and $_{135}$YPLTFGWCY$_{143}$, 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.

HLA-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_{50} values for all five peptides were in the range consistent with moderately efficient TAP transport (Table II).

The five HLA ligands were also tested for their ability to bind to their MHC class I restriction elements (Table II). For the two HLA-A2 ligands, this was done by a competition assay using immunoaffinity-purified HLA-A2 molecules. For the three HLA-B7 ligands, stabilization of HLA-B7 on the surface of HLA-B7–transfected T2 cells was determined by flow cytometry. As expected, MHC class I binding could be shown for all five peptides. The results for both the HLA-A2 and HLA-B7 ligands suggest binding affinities somewhat below that of the high affinity peptides used as positive control in the assays. Together, the TAP- and MHC-binding data would predict low to moderate efficiencies of presentation for all five of the Nef-derived HLA ligands.

Estimation of Epitope Copy Numbers Per Cell. On the basis of the results of the TAP- and MHC-binding assays, low to intermediate copy numbers of presented epitopes were predicted for all five of the peptides. The molar concentrations of the peptides recognized in acid-eluted rp-HPLC fractions were estimated in CTL assays in which each fraction was tested in serial dilutions in parallel with known molar concentrations of the same synthetic peptide. Examples are shown in Fig. 5. Total molecules of recovered peptides were calculated from the molarities, and copy numbers of the peptides per cell could then be estimated (Table II). According to these calculations, the HLA-B7 ligand Nef_{128}TPGPGVRY$_{135}$ appears to be present in high copy numbers comparable to the most efficiently presented MHC class I ligands known. Intermediate copy numbers are calculated for the second HLA-B7 ligand, Nef_{128}TPGPGVRYPL$_{137}$, whereas the remaining three peptides appear at low copy numbers. The exceptionally efficient presentation of Nef_{128}TPGPGVRY$_{135}$ cannot be accounted for by exceptionally high values for TAP transport or MHC class I binding. However, Nef_{128} TPGPGVRY$_{135}$ is by far the most abundant dual cleavage fragment in the proteasomal digest (see Fig. 4). This epitope may thus represent another example for a significant influence of proteasomes on epitope hierarchy.

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_2 termini of MHC class I ligands (1). Here we describe five naturally processed MHC class I binding peptides of HIV-1 Nef, corresponding to three different CTL epitopes. These are the first naturally processed peptides ever identified in HIV Nef. 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 $Nef_{123–152}$ (this paper) as well as of recombinant full-length Nef (Lucchiari-Hartz, M., N. Hitziger, K. Eichmann, and G. Niedermann, manuscript in preparation); (b) the NH_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.

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

Peptide	Sequence	Relative IC_{50} for TAP*	MHC class I binding	No. of MHC I ligands per cell‡
HLA-A2				
			Relative IC_{50}§	
$Nef_{136–145}$	PLTFGWCYKL	49	295	85
$Nef_{136–146}$	PLTFGWCYKLV	17	75	125
HLA-B7				
			FR‖	
$Nef_{128–135}$	TPGPGVRY	160	30	3,600
$Nef_{128–137}$	TPGPGVRYPL	195	25	840
$Nef_{135–143}$	YPLTFGWCY	304	18	80

*The unlabeled Nef peptides were used to compete the binding of ^{125}I-labeled RRYNASTEL (used at 240 nM) to TAP1/2 insect cell microsomes. The average IC_{50} of the reporter peptide in these assays was 400 nM. All values are normalized by dividing IC_{50} for test peptides by the IC_{50} 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: $Nef_{136–145}$ (69%), $Nef_{136–146}$ (74%), $Nef_{128–135}$ (88%), $Nef_{128–137}$ (95%), and $Nef_{135–143}$ (50%).

§The unlabeled Nef peptides were used to compete the binding of ^{125}I-HBV core 18–27 (6Y) to HLA-A2 molecules. The average IC_{50} of the reporter peptide in these assays was 80 nM. All values are normalized by dividing IC_{50} of unlabeled reporter peptide measured in the same assay.

‖Peptide binding to HLA-B7 class I molecules on T2-B7 cells was measured by enhanced surface expression of these molecules (see Materials and Methods). FRs obtained at 100 μM of peptide concentration are shown. FR for Nef_{198}LHPEYFKNC_{206} (negative control) = 8, for the HLA-B7–restricted self-peptide APRTVALTAL (positive control) = 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 protrude 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 NH_2 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 residues[2] of proteasomes (either tyrosine, leucine, valine, or arginine). Four ligands have small or polar aa in the NH_2-terminal (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 NH_2 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′ site (3, 4, 10, 51). The NH_2-terminal two thirds of the HLA-B7 ligand Nef_{128}TPGPGVRY_{135}, the sequence of which is identical with the NH_2-terminal flanking region of the two HLA-A2 ligands and of the HLA-B7 ligand Nef_{135}YPLTFGWCY_{143}, contains only proline, glycine, and threonine. These residues are extremely disfavored P1 residues of proteasomes (3, 10) conferring protection against proteasomal cleavage.

Four HLA ligands had low to moderate affinities for human TAP, whereas the $_{136}$PLTFGWCYKLV_{146} 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 NH_2-terminal

[2]The nomenclature for the amino acid residues of protease substrates with respect to the scissile bond is P3-P2-P1—cleavage site—P1′-P2′-P3′.

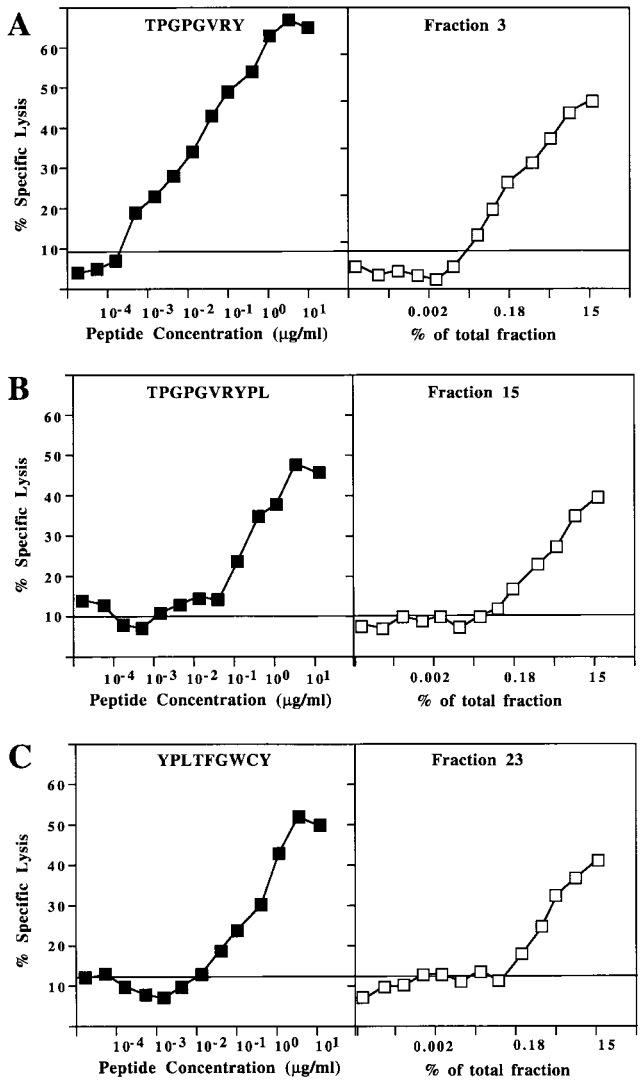

Figure 5. Titration of the defined HIV-1 Nef–derived HLA-B7 ligands (synthetic peptides) and rp-HPLC fractions of acid-eluted Nef^+ Jurkat cells. P815-B7 targets were pulsed with the indicated concentrations of peptide, and cytotoxicity assays were performed using the following Nef peptide–specific CTL lines generated from donor PR3 (see Materials and Methods): (A) $_{128}$TPGPGVRY$_{135}$-specific CTLs; (B) $_{128}$TPGPGVRYPL$_{137}$-specific CTLs; and (C) $_{135}$YPLTFGWCY$_{143}$-specific CTLs. As negative control, target cells were incubated with medium only. The E/T ratio was 100:1.

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-elongated precursor peptides were not found among the proteasomal products. One of the potential precursors of the HLA-B7–binding octamer Nef_{128}TPGPGVRY$_{135}$ 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 NH_2-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 Nef_{128} TPGPGVRYPL$_{137}$ was a single cleavage product starting with the NH_2-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 NH_2-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 L^d 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 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 KSPWFTTL peptide derived from the p15E protein of the AKV/MCF type of murine leukemia virus (53), the vesicular stomatitis virus nucleoprotein–derived epitope RGYVYQGL (59), and the peptide IPGLPLSL derived from the c-*akt* protooncogene

(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 $(MAGE)3_{271–179}$ 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 transcriptase–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, $_{128}TPGPGVRY_{135}$, 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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