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 proteaso-

1Abbreviations used in this paper: aa, amino acid(s); ER, endoplasmatic reticulum; FR, fluorescence ratio; IC50, 50% inhibition of specific binding; LAP, leucine aminopeptidase; Nt, NH2-terminally; PA28, proteasome activator 28; rp-HPLC, reversed phase HPLC; TAP, transporter associated with antigen processing; TPP II, tripeptidyl peptidase II.
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 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 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 QLESIINFEKL 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, Nef123–152, which is relatively conserved among different HIV subtypes (15). We identified two Nef-A2 and three Nef-B7 peptide ligands in this region. Unexpectedly, both Nef-A2 ligands and two of the three Nef-B7 ligands represented COOH-terminal length variants of one Nef-A2 and one Nef-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 Nef123–152. 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 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 pC’F’-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 pSBRB U6c containing the sequence encoding the whole Nef protein from HIV-1 strain LAI, under the control of the human metallothionine 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 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) 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 × 10⁶ irradiated autologous PHA (M urex 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 assessed 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 \( \mu M \) lactacystin (Dr. E.J. Corey, Harvard Medical School, Boston, MA) or 100 \( \mu M \) N-acetyl-leucyl-leucyl-norleucinal (LLnL; Sigma Chemical Co.) for 2 h before \( ^{35} \text{C} \) 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 \( ^{51} \text{Cr} \)-release assay in either the presence or absence of the inhibitors.

Synthetic Peptides. Peptides were either synthesized using solid phase 9-fluorenlymethoxycarbonyl (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; H, 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. Centrifuge 10K (Amicon Corp.) centrifugal concentrators were used for isolation of low molecular weight peptides. The ultrafiltrate suspensions (molecular weight cut off 10,000) were purified 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 T cells as described previously (4). No impurities were detected by SDS or native gel electrophoresis. Tripetidyl peptidease 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\(^{206-235}\) (Smart System [Amersham Pharmacia Biotech]) equipped with ME1 (anti-A2, -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 A2R31, 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 \( \mu \)l 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 (Micon 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 \( ^{35} \text{C} \)-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-
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 HLA class I 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-expressing cells, large-scale cell cultures of Nef-expressing cells were necessary. Because of the cytotoxic effect of Nef, attempts to prepare large-scale cell cultures of Nef-expressing cells were necessary. 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-expressing cells, large-scale cell cultures of Nef-expressing cells were necessary. Because of the cytotoxic effect of Nef, attempts to prepare large-scale cell cultures of Nef-expressing cells were necessary.

### 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

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence*</th>
<th>HLA class</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>126–138</td>
<td>NYTPGPGVRYPLT</td>
<td>B7</td>
<td>33</td>
</tr>
<tr>
<td>128–137</td>
<td>TPGPGVRYPL</td>
<td>B7</td>
<td>34, 35</td>
</tr>
<tr>
<td>130–143</td>
<td>GPGVRYPLTFGWCY</td>
<td>B57</td>
<td>36</td>
</tr>
<tr>
<td>132–147</td>
<td>GVRYLTFGWCYKLVP</td>
<td>A1, B8, B18, B49</td>
<td>37, 38</td>
</tr>
<tr>
<td>133–148</td>
<td>VRYPLTFGWCYKLVPV</td>
<td>B57</td>
<td>39</td>
</tr>
<tr>
<td>134–143</td>
<td>RYPLTFGWCY</td>
<td>B18, B49</td>
<td>37</td>
</tr>
<tr>
<td>134–144</td>
<td>RYPLTFGWCYK</td>
<td>B18, B49</td>
<td>37, 40</td>
</tr>
<tr>
<td>135–143</td>
<td>YPLTFGWCY</td>
<td>B7, B18, B49</td>
<td>37</td>
</tr>
<tr>
<td>135–144</td>
<td>YPLTFGWCYK</td>
<td>B18, B49</td>
<td>37</td>
</tr>
<tr>
<td>136–145</td>
<td>PLTFGWCYKL</td>
<td>A2.1</td>
<td>35</td>
</tr>
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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 Unef) 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 Unef 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

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**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 Nef136PLTFGWCYKL146 (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 Nef+ T1 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 fragment 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 Nef128TPGPGVRY135 (Fig. 2 A, top). Surprisingly, Nef128TPGPGVRY135-specific CTLs recognized not only fractions coeluting with the synthetic peptide Nef128TPGPGVRY135 (fraction 21/22), but also fractions coeluting with the synthetic peptide Nef128TPGPGVRY135 as well as fractions containing Nef128TPGPGVRY135. 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 Nef128TPGPGVRY135-specific CTLs not shown). These results indicate that the peptide recognized by both CTL lines was 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.

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 epitopes detected in material eluted from Nef-transfected cells were identical to those epitopes identified in the proteasomal digest.

Comparison between HLA-A2-restricted naturally processed Nef peptides and peptides derived from proteasomal degradation of the 30-mer Nef fragment 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 Nef128TPGPGVRY135 (Fig. 2 A, top). Surprisingly, Nef128TPGPGVRY135-specific CTLs recognized two rp-HPLC fractions of acid-eluted material: fraction 3 corresponding to the elution time of Nef128TPGPGVRY135, and fraction 15 corresponding to the elution time of Nef128TPGPGVRY135 (Fig. 3 A, top). Accordingly, CTLs were prepared against the smaller peptide, the octamer Nef128TPGPGVRY135, 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 Nef128TPGPGVRY135. 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 Nef128TPGPGVRY135 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 Nef128TPGPGVRY135 and Nef136PLTFGWCYKL146 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 Nef123–152 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 Nef123–152 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 Nef128TPGPGVRY135 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 Nef128TPGPGVRY135 and Nef136PLTFGWCY143 are produced upon proteasomal digestion, as both peptides are subject to destruction by the predominant cleavage site in the polypeptide Y135-P136. This major cleavage site generates the COOH terminus of the abundant HLA-B7-binding octamer Nef128TPGPGVRY135, 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 136PLTFGWCYKLV146), 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 endoplasmic reticulum (ER) (43). The IC50 values suggest a slightly higher TAP-binding affinity for the two HLA-A2 ligands than for the three
The sequence of the peptides contained in the major chromatogram obtained upon separation of the proteasomal digest of the were analyzed by mass spectrometry and Edman degradation. (A) rp-HPLC strate consumption was separated by rp-HPLC, and individual fractions were estimated in single-letter code. The Nef HLA-A2–binding ligands, nexin 123–152 was incubated with proteasomes isolated from human T1 cells (see Materials and Methods). The peptide mixture obtained after sub- peaks is indicated in single-letter code. The Nef HLA-A2–binding ligands, 135YPLTFGWCY143, are indicated by asterisks. (B) Digestion map compil- 136PLTFGWCYKL145 and 136PLTFGWCYKLV146, as well as the Nef polypeptide HIV-1 Nef123–152. A synthetic peptide corresponding to the se- digestion by 20S proteasomes of the synthetic 30-mer

**Figure 4.** Digestion by 20S proteasomes of the synthetic 30-mer polypeptide HIV-1 Nef123–152. A synthetic peptide corresponding to the sequence Nef123–152 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) An HPLC chromatogram obtained upon separation of the proteasomal digest of the 30-mer Nef123–152. The sequence of the peptides contained in the major peak is indicated in single-letter code. The Nef-128 TPGPGVRY135 is by far the most abundant dual cleavage t28, 44, 45). Nevertheless, IC50 values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45). Nevertheless, IC50 values for all five peptides were in the range consistent with moderately efficient TAP transport (28, 44, 45).

The five HLA-A2 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 deter- mined 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 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 Nef128 TPGPGVRY135 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, Nef128 TPGPGVRYPL137, whereas the remaining three peptides appear at low copy numbers. The exceptionally efficient presentation of Nef128 TPGPGVRY135 cannot be accounted for by exceptionally high values for TAP transport or MHC class I binding. However, Nef128 TPGPGVRY135 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 NH2 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 Nef123–152 (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 NH2 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 trans-ported by TAP.
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>N o. 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>PLTFGWCYKL</td>
<td>49</td>
<td>295</td>
<td>85</td>
</tr>
<tr>
<td>Nef136–146</td>
<td>PLTFGWCYKLV</td>
<td>17</td>
<td>75</td>
<td>125</td>
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<tr>
<td>HLA-B7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nef128–135</td>
<td>TPGPGVRY</td>
<td>160</td>
<td>30</td>
<td>3,600</td>
</tr>
<tr>
<td>Nef128–137</td>
<td>TPGPGVRYP</td>
<td>195</td>
<td>25</td>
<td>840</td>
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<tr>
<td>Nef135–143</td>
<td>YPLTFGWCY</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 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.

†The number of Nef ligands per Nef-T 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-labeled RRYNASTEL (used at 240 nM) to TAP1/2 insect cell microsomes. 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 80 nM. All values are normalized by dividing IC50 of unlabeled reporter peptide measured in the same assay.

Unusually, 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- tend 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 proteasomal 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 of proteasomes (either tyrosine, leucine, valine or arginine). Four ligands have small or polar aa in the NH2-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 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' site (3, 4, 10, 51). The NH2-terminal two thirds of the HLA-B7 ligand Nef128TPGPGVRY135, the sequence of which is identical with the NH2-terminal flanking region of the two HLA-A2 ligands and of the HLA-B7 ligand Nef135YPLTFGWCY143, 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 130PLTFGWCYKLV146 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

\[\text{relative IC50} = \text{IC50 (test peptide)} / \text{IC50 (unlabeled reporter peptide)}\]

\[\text{FR} = 10 \text{ ligands per cell} \]

\[\text{Relative IC50} \quad \text{for} \quad \text{TAP}\]

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

\[\text{N o. of MHC I ligands per cell} \]

\[\text{The nomenclature for the amino acid residues of protease substrates with respect to the scissile bond is P3-P2-P1—cleavage site—P1'-P2'-P3'.}\]
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 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 NYPGPGRVRY 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 Nef128 TPGPGVRYPL137 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 (4, 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 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 p89 protein of the murine cytomegalovirus 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 N\textsubscript{H}\textsubscript{2} terminus of human melanoma antigen (MAGE\textsubscript{3})\textsubscript{177-179} 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 N\textsubscript{H}\textsubscript{2} terminus (61). Together, these data suggest that proteasomes often produce the final MHC class I ligand, 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 N\textsubscript{H}\textsubscript{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 \mu M lactacystin, that from Nt-extended versions was not (11). This finding may have an alternative explanation. The peptide bond at the SIINFEKL N\textsubscript{H}\textsubscript{2} terminus is hydrolyzed efficiently by purified 20S proteasomes and proteasome-PA28 complexes (3, 4, 56). Since the SIINFEKL N\textsubscript{H}\textsubscript{2} terminus is directly preceded by glutamic acid (E), cleavage of the E\textsubscript{256}-S\textsubscript{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 N\textsubscript{H}\textsubscript{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 N\textsubscript{H}\textsubscript{2} terminus is produced by LAP, a cytosolic aminopeptidase inducible by IFN-\gamma. Small amounts of SIINFEKL were shown to be produced upon prolonged incubation of synthetic QLESIINFEKL with cytosol preparations from IFN-\gamma-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 N\textsubscript{H}\textsubscript{2}-terminal trimming) also contributes to MHC class I ligand formation. The steric constraints on peptide extensions at the N\textsubscript{H}\textsubscript{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 N\textsubscript{H}\textsubscript{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 N\textsubscript{H}\textsubscript{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 N\textsubscript{H}-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 participate in the partial downregulation of primary CD8\textsuperscript{+} 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\textsuperscript{+} 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, \textsubscript{128}TPGPGRY\textsubscript{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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