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Aminopeptidase Substrate Preference Affects HIV Epitope Presentation and Predicts Immune Escape Patterns in HIV-Infected Individuals

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Viruses evade immune detection partly through immune-associated mutations. Analyses of HIV sequences derived from infected individuals have identified numerous examples of HLA-associated mutations within or adjacent to T cell epitopes, but the potential impact of most mutations on epitope production and presentation remains unclear. The multistep breakdown of proteins into epitopes includes trimming of N-extended peptides into epitopes by aminopeptidases before loading onto MHC class I molecules. Definition of sequence signatures that modulate epitope production would lead to a better understanding of factors driving viral evolution and immune escape at the population level. In this study, we identified cytosolic aminopeptidases cleavage preferences in primary cells and its impact on HIV Ag degradation into epitopes in primary human cell extracts by mass spectrometry and on epitope presentation to CTL. We observed a hierarchy of preferred amino acid cleavage by cytosolic aminopeptidases. We demonstrated that flanking mutations producing more or less cleavable motifs can increase or decrease epitope production and presentation by up to 14-fold. We found that the efficiency of epitope production correlates with cleavability of flanking residues. These in vitro findings were supported by in vivo population-level analyses of clinically derived viral sequences from 1134 antiretroviral-naive HIV-infected individuals: HLA-associated mutations immune pressures drove the selection of residues that are less cleavable by aminopeptidases predominantly at N-flanking sites, leading to reduced epitope production and immune recognition. These results underscore an important and widespread role of Ag processing mutations in HIV immune escape and identify molecular mechanisms underlying impaired epitope presentation. The Journal of Immunology, 2012, 188: 5924–5934.

Viruses such as HIV or hepatitis C virus (HCV) evolve rapidly within hosts and populations to evade immune recognition and to spread. They have developed multiple strategies to limit the presentation of viral epitopes from infected cells to CD8 T cells (1); mutations within epitopes that prevent their binding to HLA molecules or to the TCR (2–4), mutations triggering epitope degradation (5, 6), and flanking mutations that prevent the production of epitopes of adequate length (7–9). Despite the enormous genetic diversity of HIV, population-level analyses of clinically derived viral sequences indicate that mutational escape pathways are broadly reproducible in the context of the HLA class I alleles expressed by the host (10–13). Indeed, a large number of HLA-associated polymorphisms have been identified across HIV, including many flanking epitope boundaries (14–17).

Epitopes displayed by MHC class I (MHC-I) complexes are derived from intracellular degradation of proteins. Proteins or incomplete translation products are degraded by the proteasome in fragments of various lengths that are further degraded by endopeptidases and aminopeptidases (18, 19). N-extended versions of epitopes can be shortened by endopeptidases such as nardilysin (20), insulin-degrading enzyme (21), or by cytosolic endoplasmic reticulum (ER)-resident aminopeptidases. Leucine aminopeptidase (LAP) (22, 23), puromycin-sensitive aminopeptidase (24), ERAP1 (25, 26), and ERAP2 (27) play variable and complementary roles in producing or destroying epitopes. ERAP1 knocked-out mice infected with lymphocytic choriomeningitis virus present different CD8 T cell responses compared with wild-type (WT) animals, highlighting the important role of aminopeptidases in epitope production (28, 29).

Cleavable residues have been identified for aminopeptidases in immunized cells (30–32), but the impact of these flanking residues on immune escape was not assessed. Artificial or natural
mutations flanking epitopes on the N-terminal side and altering epitope presentation have been identified for hepatitis B (33), influenza (34), and murine retroviruses (35), although the role of aminopeptidases in the production of these epitopes was not assessed. We identified an HLA-restricted mutation flanking an HIV epitope that prevents the trimming of N-extended epitope by ERAP1 and impairs recognition of infected cells by epitope-specific CTL (7). We reasoned that definition of well-cleavable and noncleavable motifs would allow us to identify Ag processing mutations in highly variable viruses at the population level.

After establishing the amino acid cleavage preferences of human PBMC, CD4 T cells, and monocytcs, we demonstrate a strong correlation between the cleavability of N-flanking residues, the production of the adjacent epitope, and the endogenous processing and presentation of the epitope to CD8 T cells. These in vitro findings were supported by in vivo population-level analyses of HLA-associated polymorphisms from viral clinical isolates identified through analysis of HIV sequences from 1134 patients with known HLA class I types. We show that at N-flanking sites of known CTL epitopes, HLA-restricted immune pressures in vivo drive the selection of residues that are less cleavable by aminopeptidases, thus reducing epitope production and presentation. To our knowledge, these results provide the first demonstration of sequence signatures defined at the population level that lead to impaired epitope presentation and impaired immune recognition, supporting a critical role of Ag processing mutations in immune escape in HIV infection.

Materials and Methods

Peptides and reagents

Highly purified peptides (>98% pure) were purchased from Massachusetts General Hospital peptide core facility or from Biosynthesis. All chemicals were purchased from Sigma and fluorogenic substrates from Enzo Life Sciences. Actin (Abcam), and absence of ER markers. Cytosolic extracts were also checked for the absence of contamination by lysosomes through the measurement of cathepsin activities with a pan-cathepsin fluorogenic substrate (data not shown and Refs. 36, 37).

Aminopeptidase activity measurement

Aminopeptidase activities in 3 μg cell extracts were measured in 96-well plates with 12.5 μM X-aml fluorogenic substrate. Fluorescence emission was measured in the presence or absence of extracts at 37°C every 5 min during 1 h on a Victor-3 plate fluorescence reader (PerkinElmer, Boston, MA). Fluorescence emission after peptide cleavage is proportional to the activity. The specificity of the reaction was checked by preincubation of extracts with inhibitors of proteasomes (MG132 10 μM, lactacystin 10 μM), aminopeptidases (bestatin 12 μM, puromycin 10 μM), metallopeptidase (1,10-phenanthroline 1 μM), tripeptidylpeptidase II (butyrobetaine 330 nM), cysteine proteases (E64 50 μM), and cysteine/serine aspartic protease (leupeptin 10 μM). At each time point, fluorescence values in the absence of extracts were subtracted to that measured in the presence of extracts. Substrate cleavage kinetics was reported as the maximum slope (i.e., initial velocity) of this graph.

In vitro epitope degradation and peptide antigenicity assay

Peptides (8 nmol) were degraded with 40 μg PBMC cytosol as described previously (36, 37). Peptides present in the digestion mix at designated times were purified by TCA precipitation and identified by mass spectrometry. The mix was diluted to 400–1600 fmol in 80% water, 15% ammonium hydroxide, 5% trifluoroacetic acid (v/v) and loaded on a C18 column (Nano-LC Ekisgent) and electrospayed on an Orbitrap Discovery mass spectrometer (Thermo). Each peptide present in the mix, the area of the peak it generated, and the proportion of peptides at each time point were calculated with Proteome Discoverer (Thermo) (Harvard Medical School mass spectrometry facility and in-house mass spectrometer). The intensity of a peak generated by a given peptide is proportional to the amount of peptide (Supplemental Fig. 2A). Each degradation time point was run on the mass spectrometer at least twice. Repeated injections of degradation products show strong reproducibility (Supplemental Fig. 2B).

Antigenicity of degradation products

Peptides present in the digestion mix were purified, diluted in RPMI 1640 without serum (R+), and pH was readjusted to 7.4. Cells labeled with [3H]Cr were pulsed with 0.02 μCi/ml digestion products without serum and used as targets in killing assays with epitope-specific CTL clones at a 4:1 ratio as in Ref. 37. Lysis percent was calculated as follows: (cpm from CTL-mediated Cr release – spontaneous Cr release)/maximum (induced with Triton X-100 2.5%) – spontaneous Cr release) × 100. Lysis percent of cells pulsed with degradation products was compared with those of HLA-matched B cells pulsed with undigested long peptides or optimal epitopes at concentrations ranging from 0 to 0.4 μg/ml.

mRNA preparation and transfection for epitope presentation

HIV-1 p24 synthetic genes were purchased from GenScript. Each construct included a T7 promoter, a Kozak sequence, Ag open reading frame, stop codon, and a polyA signal, and synthetic mRNA were transcribed from linearized plasmid templates, quantitated using a ND-1000 spectrophotometer as in Ref. 38. The size and purity of mRNA preparations were confirmed with the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Three million B cells were electroporated using the following conditions: 100 V, 250 μF, Bio-Rad GenePulser X Ella (Bio-Rad, Hercules, CA). Transfection efficiency was >80% (38). Twenty hours later, cells were used as targets in the [3H]Cr release assay.

Cohort and identification of HLA-restricted mutations

We used published, phylogenetically corrected methods (15) to identify HLA-associated viral polymorphisms in a cohort of 1134 chronically HIV-infected, treatment-naive individuals from British Columbia, Canada, for whom HLA class I types and nearly full genome H1V-1 sequences were characterized (11). Analyses of all HLA class I alleles and their restricting epitopes were performed at two-digit resolution. We identified HLA-associated polymorphisms occurring inside or within ±3 residues of all optimally defined CTL epitopes across the HIV-1 proteome except gp120 (39), in the context of the restricting HLA only. A maximum likelihood phylogenetic tree was constructed from the HIV sequences in the data set, and a model of conditional adaptation was inferred for each observed amino acid at each codon (14, 15). In this model, the amino acid is assumed to evolve independently down the tree until it reaches the terminal branch tips. On each tip, the selection pressure arising from HLA-associated immune pressures is directly modeled using a stochastic additive process where each HLA allele is treated as a binary variable. The p value is computed using the likelihood ratio test. Multiple comparisons are addressed using q values, which estimate the expected proportion of false positives among results deemed significant at a given threshold (40). For example, at q ≤ 0.2, we expect 20% false positives among identified associations. Using this approach, we are able to identify two types of HLA-associated polymorphisms: 1) amino acids significantly depleted in the presence of the HLA allele in question (the “nonadapted” form) and 2) amino acids significantly enriched in the presence of the HLA allele in question (“adapted” or “escape” form) (14, 15). At a q value of 0.2, we identified a total of 102 HLA-associated polymorphisms occurring at positions ~3 through 3 of known CTL epitopes for which a specific “adapted” form was identified for the restricting HLA, thus allowing us to calculate α aminopeptidase scores between “nonadapted” and “adapted” forms. These escape pathways included 8, 8, and 10 HLA-associated polymorphisms occurring at codons 1, 2, and 3 of known CTL epitopes, respectively, and 21, 29, and 25 HLA-associated polymorphisms occurring at positions 1, 2, and 3 of known CTL epitopes, respectively. Out of 102 mutations, 14 mutations at position 2 were pre-
dicted to decrease HLA binding affinity by more than 10-fold (NetMHC3.2; www.cbs.dtu.dk/services/NetMHC/) (41); these were removed from analysis as they likely represented escape mutations whose main mechanism of action is abrogation of peptide–HLA binding. The published B57-associated A146P escape mutation occurring at position −1 of the B57-ISW9 (Gag) epitope (7)—primarily selected by HLA-B5703, whereas our cohort included mainly HLA-B5701 individuals—was added to the list, bringing the final total of investigated HLA-associated escape pathways to 102.

Statistical analysis
Empirical data were analyzed using GraphPad Prism version 5.

Results
Cytosolic aminopeptidase substrate preferences in human primary cells
We first aimed to establish aminopeptidase substrate preferences in human PBMC, CD4 T cells, and monocytes. Fluorogenic substrates composed of an amino acid (X, where X represents any amino acid) coupled to coumarin derivative (X-amc) were incubated with PBMC cytosol for 60 min, and fluorescence emission was monitored every 5 min as done in Ref. 36 (Fig. 1A). As previously reported in immortalized cells, leucine-amc was very efficiently cleaved by cytosolic aminopeptidases, whereas tyrosine-amc was cleaved less efficiently (22, 30, 32). The specificity of substrate cleavage was checked by preincubation of extracts with inhibitors of aminopeptidases (bestatin, puromycin), metallopeptidases (1,10-phenanthroline), proteasome (MG132 or lactacystin), TPPII (butabindide), cysteine proteases (E-64), or cysteine/proteases (leupeptin) (Fig. 1B). For each substrate, the hydrolysis kinetics are measured as the maximum slope of fluorescence emission during a 1-h experiment after subtraction of fluorescence in the absence of extracts. One hundred percent represents the maximum slope of Leu-amc and Tyr-amc (928 and 316, respectively). It was reduced by >70% in the presence of inhibitors of aminopeptidases or metallopeptidases and much less (<22%) by inhibitors of proteasome, TPPII, and serine/cysteine proteases.

We compared the hydrolysis of 20 X-amc substrates in PBMC cytosol from five different donors. The hydrolysis kinetics of aminopeptidase sp. act. is calculated after subtraction of fluorescence in the presence of bestatin (Fig. 1C). Leu-amc was the most efficiently cleaved substrate, and its hydrolysis rate was used as 100% to rank other amino acids. Beside Leu-amc, nine X-amc were cleaved with variable efficiency: three amino acids (M, K, F) formed very efficiently cleavable bonds (slopes 77–85% of that of Leu-amc), whereas six amino acids (Y, A, R, C, W, I) formed bonds hydrolyzed at lower rates (11–53%). Ten amino acids (V, T, P, N, Q, D, E, G, H, S) formed bonds that were poorly or not cleaved by aminopeptidases (0–4.9%). The split between well-cleavable and poorly cleavable residues is in accordance with published results in immortalized cells (31, 32). However the best aminopeptidase substrate in primary human cells was Leu-amc and not Lys-amc.

We had previously reported that monocytes have higher cytosolic aminopeptidase activities than CD4 T cells using Leu-amc substrate (36). Whether this difference remains for other amino acids is unknown. We compared the hydrolysis of nine X-amc bonds representing highly to poorly cleaved residues in cytosolic extracts from PBMC, CD4 T cells, or monocytes sorted from the same four donors (Fig. 1D). The ranking of the nine amino acids was equivalent between PBMC, CD4 T cells, and mono-
We aimed to determine how good or poor aminopeptidase substrates affect the production of adjacent epitopes. We first identified HIV epitopes whose production requires aminopeptidase trimming (Supplemental Fig. 1). HLA-B*57+ B cells were transfected with RNA encoding HIV p24 Gag as in Ref. 38 and used as targets in a killing assay with CTL specific for B57-ISW9 (ISPRTLNAW; aa 15–23) or B57-KF11 (KAFSPEVIPMF; aa 30–40), two epitopes targeted by HLA-B*57 individuals during chronic HIV infection (42). Preincubation of cells with a proteasome inhibitor or by targeted N-flanking residues alter the trimming of N-extended epitopes in vitro.

We compared the cytosolic processing of KF11 from N-extended peptides containing the WT N-extension (VEE) or modified N-extension including good (VLL, VMM, VFF, VKK) or poor (VII, VSS, VHH, VVV) aminopeptidase substrates. The degradation products generated in 1 h were identified by mass spectrometry as we previously described (5, 36, 37). The peak area corresponding to a sequence of given mass and charge is proportional to the amount of corresponding peptide (Supplemental Fig. 2). We compared KF11 peak area generated from each mutated peptide over that of KF11 produced from the WT sequence (Fig. 2A). All four highly cleavable flanking motifs led to increased KF11 production (2.7- to 9.7-fold). In contrast, the introduction of motifs as poorly cleavable as the WT sequence reduced or hardly affected the production of KF11. We also measured the production of FF9, another B57-restricted epitope within KF11 located two residues downstream of the KF11 start site (43). The production of FF9 was also strongly increased for peptides containing good aminopeptidase substrates. These results show that changes one to four residues upstream of a peptide can affect its production. To rule out any differences in degradation products recovery, mass spectrometer injection, or peptide detection, we quantified shorter peptides common to all sequences (i.e., included in KF11). The production of SPEVIP from mutated sequences was 0.8- to 1.4-fold that of WT sequence, showing that changes one to four residues upstream of a peptide can affect epitope processing and presentation.

We compared the cytosolic processing of KF11 from N-extended peptides containing the WT N-extension (VEE) or modified N-extension including good (VLL, VMM, VFF, VKK) or poor (VII, VSS, VHH, VVV) aminopeptidase substrates.

**FIGURE 2.** Flanking residues drive the amount and antigenicity of HIV peptides produced in PBMC cytosol. (A) Three-residue-extended KF11 peptides (KAFSPEVIPMF; aa 30–40 in p24) with double mutations flanking KF11 (WT VEE or mutants LL, MM, FF, KK, II, SS, HH, DD, VV) were degraded for 1 h in PBMC cytosol. Cleavable motifs appear in boldface, and poor substrates are italicized. Peptides KF11 (black bars), FF9 (gray bars), and SPEVIP (white bars) were identified by mass spectrometry. The intensity of the peak of the three peptides was compared between WT and mutants. Representative of three experiments. (B) Correlation between the fold increase production of KF11 (open circles) and FF9 (black triangles) and the relative PBMC aminopeptidase activity. The efficiency of cleavage of an amino acid established with fluorogenic substrates (Fig. 1C) correlated with the production of flanking epitope KF11 (R = 0.64; p = 0.0026) and downstream epitope FF9 (R = 0.83; p < 0.0001) (Fig. 2B), suggesting that changing aminopeptidase substrates affects epitopes immediately adjacent to or two residues downstream of the residue being cleaved. We also assessed the production of KF11 and FF9 from peptides containing a single flanking mutation toward better (L, M, F) or poor (I, D, H, V, S, T) aminopeptidase substrates. Mutations toward a good substrate increased KF11 production albeit less efficiently than with a double mutation (up to 6.2-fold), albeit less efficiently than with a double mutation.
and replacement of E by another poorly cleavable residue did not alter epitope production (0.9- to 1.5-fold difference) (Supplemental Fig. 3A). The only exception was poorly cleavable residue S for which KF11 production increased by 5.6-fold.

As an independent read-out of epitope production, we assessed the antigenicity of the degradation products generated over 1 h as previously performed (5, 36, 37). Purified peptides were used to pulse an HLA-B57+ cell line used as targets in a [51Cr]-based killing assay (Fig. 2C). At low peptide concentration, extended KF11 peptide such as VEE-KF11 did not yield a KF11 CTL response (lysis <2%); however, as the peptide N-extension was trimmed, the antigenicity of the degradation products increased. Degradation products of VEE-KF11 became antigenic after a 10-min degradation, and antigenicity plateaued at 20.6–22.8% for products generated in 30 to 60 min. In contrast, for peptides where KF11 was preceded by good aminopeptidase substrates (FF, KK, LL, MM; Fig. 2C, left panel), antigenic products were generated faster and in higher quantity (maximum antigenicity around 40–50% compared with 22.8% for WT products). For peptides encompassing poor aminopeptidase substrates (Fig. 2C, right panel), the antigenicity of degradation products from three peptides (II, DD, VV) was comparable to that of WT products (with VV generating more antigenic products at time 60 min) as expected from the in vitro production of KF11. However, two peptides with poor aminopeptidase substrates (SS and HH) efficiently generated antigenic products (51.6 and 54% at 60 min for HH and SS, respectively). Out of 10 amino acids tested, 8 behaved as hypothesized, whereby poor/good aminopeptidase substrate flanking epitope led to low/high in vitro epitope production from N-extended epitopes and low/high antigenicity of degradation products. Similarly for peptides carrying a single flanking mutation, the antigenicity of the degradation products was increased for peptides including good aminopeptidase substrates and correlated with KF11 production measured by mass spectrometry (Supplemental Fig. 3B). These results suggest that the two residues flanking KF11 drive the efficiency of epitope production.

The efficiency of endogenous epitope processing and presentation is driven by N-flanking motifs

We confirmed the relevance of good and bad aminopeptidase substrates during the endogenous processing and presentation of KF11 epitope by cells. HLA-B57 cells were transfected with RNA vectors expressing p24 in which KF11 is flanked by various motifs (Fig. 3). In this experimental setting, both cytosolic and ER-resident aminopeptidases can contribute to the production or degradation of KF11. We monitored the presentation of KF11 and of TW10, another B57-restricted epitope located in Gag p24 (Fig. 3A). We hypothesized that enhanced production of KF11 due to optimal flanking residues would lead to its improved presentation and an increase in the ratio of KF11/TW10 peptide presentation.

The endogenous processing and presentation of WT p24 led to efficient killing by TW10-specific CTL (58.5%) and KF11-specific CTL (24.2%), a 2.4-fold difference in CTL lysis. p24 containing II-KKF11, another poorly cleavable motif, led to similar lysis rates by each clone (23.8% and 45% by KF11 and TW10 CTL respectively, a 1.9-fold difference in lysis percent). In contrast, expression of p24 with LL-KF11, an efficiently cleavable motif, led to equivalent lysis by KF11 and TW10 clones (41.7% and 37.7%, a 0.9-fold difference in lysis), suggesting enhanced presentation of KF11 when preceded by a cleavable motif. To take into account possible differences in peptide avidity for HLA-B57 and TCR and variations in transfection efficiency among variants, we measured an equivalent amount of peptide presented by transfected cells by...
HIV-infected individuals (7). We performed in vitro degradation of N-extended peptides (HQ-A-ISW9, HQP-ISW9 A146P) in PBMC cytosol and identified the degradation peptides produced over 1 h by mass spectrometry (Fig. 4A, Supplemental Fig. 4). The kinetics of degradation of HQ-A-ISW9 and HQP-ISW9 were similar (Fig. 4A, left panel). The degradation of HQ-A-ISW9 led to the production of A-ISW9 and ISW9 (up to 4.6% of total peptides). In contrast, the degradation of HQP-ISW9 led to the accumulation of QP-ISW9 (up to 11% of total amount of peptides) and no production of A-ISW9 or ISW9, likely due to the incapacity of cytosolic aminopeptidases to cleave the Q-P bond. We compared the antigenicity of the degradation products generated from the degradation of HQ-A-ISW9 or HQP-ISW9 (Fig. 4B). It reached up to 19% lysis at 10 min for WT where it was at 2% for WT p24 containing KF11 led to a 3.9-fold increase of KF11 presentation compared with presentation of KF11 from WT, suggesting that replacement of poorly cleavable flanking motif by other poor substrates did not significantly change KF11 presentation. We showed a strong correlation between endogenous processing and presentation of KF11 and the cleavability of the flanking residues (\( p = 0.006, R = 0.878 \)) and the in vitro production of KF11 (\( p = 0.0022, R = 0.812 \)). This demonstrates that the capacity of aminopeptidases to cleave epitope flanking residues plays a critical role in the amount of epitope produced and subsequent recognition by epitope-specific CTL. It also provides strong rationale for the use of in vitro epitope processing as a way to identify motifs altering epitope production in cells.

**FIGURE 4.** An HLA-restricted epitope-flanking mutation toward a poorly cleavable aminopeptidase motif impairs epitope production and presentation. (A) Peptide corresponding to HLA-B57-ISW9 with three-residue extension with WT (HQ-A-ISW9, black circles) or mutated HQP-ISW9 (gray triangles) was degraded in PBMC cytosol. The relative amount of 3-, 2-, 1-extended, and epitope ISW9 (left to right) was identified by mass spectrometry over 60 min. The relative amount of each peptide in the total mix of degradation products is quantified at each time point. Average of two degradation experiments run twice on a mass spectrometer. Two additional degradation experiments with less cytosol gave similar results albeit with slower degradation rates (not shown). (B) Degradation products from HQA-ISW9 (black circles) or HQP-ISW9 (gray triangles) of each time point were pulsed onto HLA-B57 cells used as targets in a chromium assay with ISW9-specific CTL. Average of three killing assays run in triplicate. (C) HLA-B57 cells were transfected with RNA encoding WT p24 (black bars), p24 with A146P mutation flanking ISW9 (gray bars), or GFP (white bars) and used as targets in a chromium-based killing assay with ISW9-, KF11-, or TW10-specific CTL (three epitopes located in p24 and restricted by HLA-B57). Epitope flanking sequences indicated above bars. Average of three transfections; killing assays run in triplicate.

Because aminopeptidases play a critical role in trimming epitope precursors and have marked substrate preferences, HLA-restricted mutations occurring near the N terminus of epitopes could significantly impact epitope presentation. We hypothesize that there could be two ways to reduce or eliminate epitope production in vivo. First, selection of N-flanking mutations representing poor aminopeptidase substrates could lead to reduced or impaired trimming of epitopes. Conversely, selection of mutations within the epitope toward better aminopeptidase motifs could lead to “over-trimming” of the epitope.

We analyzed the impact of nine specific HLA-restricted flanking or intraepitopic mutations (positions –3 through 3) creating motifs predicted to be more or less cleavable by aminopeptidases on HIV epitope production (Figs. 4, 5).

The HLA-B57–restricted alanine 146 to proline (A146P) escape mutation flanking Gag p24 epitope ISW9 whose processing is aminopeptidase-dependent is frequently observed in HLA-B57+ HIV-infected individuals (7). We performed in vitro degradation of N-extended peptides (HQA-ISW9, HQP-ISW9 A146P) in PBMC cytosol and identified the degradation peptides produced over 1 h by mass spectrometry (Fig. 4A, Supplemental Fig. 4).
HQP-ISW9, a 9-fold difference in equivalent ISW9 peptide production in accordance with the lack of production of ISW9 from HQP-ISW9. The production of ISW9 and antigenicity decreased later on, likely due to rapid cytosolic degradation of ISW9 (5).

We assessed the complete endogenous processing and presentation of HLA-B57–restricted epitopes by RNA transfection of WT or A146P p24 in HLA-B57+ B cells and assessed the presentation of three HLA-B57–restricted epitopes, ISW9, KF11, and TW10 (Fig. 4C). In this case, both cytosolic and ER aminopeptidases may contribute to epitope processing. Cells transfected with WT or A146P p24 were equally recognized and killed by CTL specific for TW10 or KF11, showing that transfection and expression of WT or A146P p24 were similar. Mutation A146P flanking ISW9 reduced killing of transfected cells by ISW9-specific CTL by 5-fold (9.4 versus 50.6%), showing that a mutation creating a poorly cleavable motif (d 2 50) flanking an epitope led to lack of production and presentation of the epitope and reduced killing by epitope-specific CTL.

We assessed the impact of four additional mutations upstream of HIV epitopes (Fig. 5A): HLA-B08–restricted L to I mutation at position 23 of B08-FL8 in Nef, HLA-B40–restricted T to I at position 22 of B40-IL9 in RT, HLA-A11–restricted A to V mutation at position 21 of A11-T19 in p17, and HLA-A03/11-restricted A to G at position 21 of AK9 in Nef. For each sequence, we assessed the disappearance of WT and mutated three-residue-extended peptide and the appearance of the epitope in a 1-h cytosolic degradation. Mutation three residues upstream of B08-FL8 in Nef did not affect epitope production, and mutation at position 22 of B40-IL9 slightly reduced epitope production. Mutations directly flanking epitopes A11-T19 and A03/A11 AK9 led to slower degradation of the N-extended peptides and reduced epitope production by 2.3- and 10.3-fold, respectively.

Similarly, we assessed the impact of four HLA-restricted intraepitopic mutations toward better aminopeptidase substrates (Fig. 5B): HLA-Cw15–restricted R to K mutation at position +1 of RL9 in Nef, HLA-B57–restricted I to L mutation in p24 B57-ISPRTLNAW in p24, HLA-B27–restricted R to K at position 2 in integrase, and HLA-A03–restricted V to L mutation at position 2 in A03-AK9 in Nef. Whereas the Cw15-restricted mutation increased epitope production by 2.2-fold, the B57-, B27-, and A03 mutations at positions 1 or 2 reduced the production of the epitope by 2.4-, 7.8-, and 3.0-fold, respectively.

Altogether, these results provide proof of concept that published escape mutations toward a poorly cleavable motif at epitope flanking sites, and toward more efficiently cleavable motifs within the epitope itself, reduce production of the relevant epitope. These results suggest that Ag processing mutations affecting peptide trimming occur frequently in vivo and that they can be predicted.

Aminopeptidase substrate preferences define HLA-restricted Ag processing mutations at the population level

To address further whether alterations in aminopeptidase substrate preferences could represent a major mechanism underlying HLA-driven immune escape pathways in vivo, we analyzed linked HLA class I types and nearly full-genome HIV-1 sequences in a cohort of 1134 individuals infected with HIV-1 clade B ([14, 44] and Z.L. Brumme, unpublished observations). We aimed at identifying HLA-restricted mutations occurring in the three N-flanking residues (positions 2 through 1) and in the first three residues of the epitope (positions 1 through 3) for all optimally described CTL epitopes in HIV-1 (except envelope gp120) (45). This anal-

![FIGURE 5. HLA-restricted flanking mutations toward less cleavable motifs or intraepitopic mutations toward more cleavable aminopeptidase motifs reduce epitope production. (A) Peptides including an HIV epitope with a three-residue N-terminal extension corresponding to WT sequences (black bars) or with an HLA-restricted mutation at positions 1, 2, 1 (gray bars) were degraded in PBMC cytosol for 60 min. All degradation peptides were identified by mass spectrometry, and the relative amount of the three-residue-extended peptide and epitopes was calculated. Names, sequences, and HLA restriction of each epitope are indicated below each panel. N-extended sequences, mutations, and their locations are indicated above each panel. (B) Same as (A) with HLA-restricted mutations located at positions 1 or 2 within epitopes. Average result of three mass spectrometry injections of one representative cytosolic degradation.](http://www.jimmunol.org/Downloadedfrom/5930%20SEQUENCE%20SIGNATURES%20OF%20HIV%20Ag%20PROCESSING%20MUTATIONS)
ysis revealed a total of 102 distinct escape mutations significantly linked to an HLA allele and occurring at positions −3 through +3 of a published CTL epitope. Fourteen mutations at positions affecting binding to MHC-I by more than 10-fold were excluded from this analysis as impaired HLA binding may be the main escape mechanism at this site. For each escape mutation, we calculated the difference ($\delta$; $\Delta$) between the cleavage capacity of the epitope (or N-extended epitope precursor) containing the adapted (escape mutated) residue and that containing the nonadapted (usually consensus B) residue, based on the aminopeptidase rankings established in Fig. 1C. A negative $\delta$ is indicative of a mutation toward a worse aminopeptidase substrate, whereas a positive $\delta$ indicates a mutation toward a better aminopeptidase substrate. At positions −3 through 3 of known CTL epitopes, a broad range of $\delta$ scores were observed (Fig. 6A). Escape mutations occurring at position −1 of known CTL epitopes significantly deviated from a theoretical median $\delta$ score of 0 (Wilcoxon signed-rank test $p = 0.018$) and exhibited a strong trend toward poorer aminopeptidase substrates with 9 mutations out of 11 (81.8%) exhibiting negative deltas (mean = −31.3; range = −22.6 to −85). Within epitopes, mutations did not significantly deviate from a median $\delta$ score of 0, although position 2 presented the highest proportion of escape mutations toward more cleavable residues [8 out of 15 (53%) mutations not affecting MHC-I binding]. The comparison of mutations restricted by protective HLA alleles (HLA B57, B58, B27) versus neutral or nonprotective alleles also show similar, statistically significant tendencies toward less cleavable residues at position −1 (Fig. 6B). These results suggest that regardless of the HLA allele restricting the mutation, evolution toward residues poorly cleavable by aminopeptidases at N-flanking residues appears to be a hallmark of immune escape in HIV.

Peptides translocated into the ER can be further trimmed by ER aminopeptidases with defined substrate preferences (30–32). The comparison of the hydrolysis rate of amino acids in the PBMC cytosol established in this study and that of ER microsomes from various cell lines established by Schatz et al. (32) showed strong correlation ($p = 0.0021$), in accordance with some shared cleavable residues such as M, K, L and shared poorly cleavable substrates such as P, D, E (Fig. 6C). We analyzed the effect of HLA-restricted mutations on ER aminopeptidase hydrolytic rates. Similarly to trends observed for cytosolic peptidases, mutations at position −1 significantly evolved toward less cleavable residues by ER aminopeptidases ($p = 0.018$) (Fig. 6D), suggesting that mutations could impair epitope processing in the cytosol and/or in the ER for peptides reaching this compartment. Our results support the notion that alterations in aminopeptidase substrate potential represents a key mechanism underlying in vivo HLA-restricted immune escape pathways identified at the population level.

**Discussion**

Identifying viral mutations that will impair epitope presentation to immune cells is critical to understand reasons for immune failure in chronic infections and to devise better therapeutic strategies. Although recent improvements in DNA sequencing and computation have facilitated the identification of immune-driven mutational pathways in rapidly evolving viruses such as HIV and HCV, the molecular mechanisms underlying these evolutionary pathways remain largely unknown. Understanding immune escape at

**FIGURE 6.** HIV evolution at the population level toward poorly cleavable flanking residues and more cleavable residues within epitopes. (A) HLA-associated escape pathways located at positions −3 (diamond), −2 (inverted triangles), −1 (squares) flanking epitopes or at position 1 (circles), 2 (triangles), or 3 (stars) were identified in all optimally defined CTL epitopes across the HIV-1 proteome (except gp120) in a cohort of 1134 chronically HIV-infected, antiretroviral-naïve individuals. A total of 88 HLA-restricted escape pathways (after exclusion of 14 mutations affecting binding to MHC-I) were identified. For each given HLA-restricted escape pathway, we calculated the difference ($\delta$; $\Delta$) between the aminopeptidase cleavage capacity of the adapted (escape mutated) residue and that of nonadapted (usually consensus B) residue, based on the ranking established in Fig. 1C. A negative $\delta$ is indicative of a mutation toward a worse aminopeptidase substrate, whereas a positive $\delta$ indicates a mutation toward a better aminopeptidase substrate. Wilcoxon signed-rank test for deviation from a theoretical median $\delta$ score of 0 at position −1 ($p = 0.018$). (B) Same as in (A) except mutations are compared only at positions −1, 1, and 2 for HLA alleles associated with protection (HLA B27, B57, B58) (left, gray symbols) or neutral or associated with progression (right, open symbols). (C) Comparison of amino acids hydrolysis rates by PBMC cytosolic aminopeptidase established in Fig. 1 and ER aminopeptidases established by Schatz et al. (32). Spearman correlation. (D) Similar to (A) except that the difference ($\delta$; $\Delta$) between the aminopeptidase cleavage capacity of the adapted (escape mutated) residue and that of nonadapted residue is based on the ranking of ER aminopeptidases established by Schatz et al. (32). Wilcoxon signed-rank test for deviation from a theoretical median $\delta$ score of 0 at position −1, $p = 0.018$. 

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the molecular level is not possible without first achieving clear understanding of basic mechanisms of epitope presentation. In this report, we show that cytosolic aminopeptidases in the class I Ag processing pathway cleave substrates with varying efficiency and that we can use this hierarchical pattern to define sequence signatures leading to impaired HIV epitope production. Importantly, these signatures were consistent with in vivo immune-mediated escape pathways identified from patient-derived HIV sequences at the population level.

The degradation of proteins into epitopes presented by MHC-I relies on multiple peptidases located in several subcellular compartments. Aminopeptidases play a critical role in the postproteasomal trimming of peptides into epitopes either in the cytosol or in the ER. Aminopeptidases located in the cytosol and in the ER share many cleavage preferences despite some ranking differences among compartments. As degradation peptides traffic from the cytosol to the ER and—indeed for proteins in the exogenous processing pathway—from endo-lysosomes to ER or from endo-lysosomes to cytosol and ER before cross-presentation, mutations leading to altered peptide trimming by aminopeptidases may provide a common way to reduce epitope presentation regardless of the path followed by the Ag. Mutations with the strongest effect on aminopeptidase cleavage capacity appeared at position 1 in the cytosol and in the ER. HIV epitopes linked to these HLA-associated mutations may mainly rely on aminopeptidase trimming, whereas epitopes associated with mutations at other positions may involve trimming by peptidases with different cleavage preferences. Aminopeptidase-dependent epitope B37-ISW9 is associated with mutations at position 1 (alanine to proline) preventing epitope production and at position 1 (isoleucine to leucine) enhancing peptide degradation. Additionally, aminopeptidases can cleave one or two residues at a time, thus not all mutations would have the same impact on aminopeptidase-mediated trimming. For other mutations, impaired aminopeptidase trimming may not be the main force driving for viral evolution. Proteasomes and endopeptidases such as nardilysin also contribute to the processing of HIV epitopes, and modifying their cleavage sites may also impair epitope processing (20, 46). Because different peptidases have different specificities with regards to lengths of the substrates and cleavable motifs, we expect that specific viral mutations will affect the activity of each class of enzymes that contribute to Ag processing and may explain some of the other HLA-restricted mutations. A comprehensive analysis of signatures of Ag processing mutations will require a better understanding of cleavage preferences of all peptidases. Proteasome-mediated destruction of HIV epitopes, and modifying their cleavage sites may also impair epitope processing (20, 46). Because different peptidases have different specificities with regards to lengths of the substrates and cleavable motifs, we expect that specific viral mutations will affect the activity of each class of enzymes that contribute to Ag processing and may explain some of the other HLA-restricted mutations. A comprehensive analysis of signatures of Ag processing mutations will require a better understanding of cleavage preferences of all peptidases. Proteasome-mediated destruction of HIV epitopes has been identified for a few HLA-associated mutations (6, 47). Peptides corresponding to HIV epitopes of optimal size are also subjected to cytosolic degradation prior to loading onto MHC-I. We have identified multiple intraepitopic HLA-restricted mutations that render HIV epitopes more sensitive to cytosolic degradation (5). These mutations create cleavage sites for cytosolic peptidases and were located at various positions throughout the epitope. Out of 25 mutated epitopes, 21 (84%) peptides saw their cytosolic stability reduced by 22–99% relative to WT, suggesting that multiple intraepitopic mutations lead to peptide degradation before loading onto MHC-I molecules. It is difficult—just from the location of a given HLA-restricted mutation—to determine if a mutation will affect epitope production, binding to MHC-I or to the TCR or be a compensatory mutation (3, 4). However, our data provide a way to identify many mutations impairing epitope production and suggest that Ag processing mutations can occur at many positions within or outside of epitopes. It takes multiple trimming steps to produce an epitope from a protein, providing multiple opportunities for viral mutations outside epitopes that may interfere with its trimming and production. In addition, a typical epitopic sequence contains 8 to 11 residues that could potentially be mutated to produce more cleavable targets and lead to epitope destruction in just one step. Even if HIV evolution is limited by structural and fitness constraints, Ag processing mutations provide multiple redundant options for HIV to escape immune recognition. Other variable viruses such as HCV also evolve Ag processing mutations to escape immune responses (3, 48, 49) although the contribution of flanking and intraepitopic mutations to immune failure remains to be clarified.

We showed that the higher aminopeptidase activity detected in monocytes was mainly due to higher expression and activity of leucine aminopeptidase or closely related aminopeptidases. This suggests the existence of cell type-specific expression of aminopeptidases as evidenced previously for a macrophage-specific serine protease (50). Differences in the pattern of expression and/or activities of aminopeptidases between different cell types may affect the kinetics and stoichiometry of epitope production within different cell types as we showed for CD4 T cells and monocytes (36) and should be assessed for additional cell types of interest to viral epitope presentation. Because both CD4 T cells and macrophages can be recognized by HIV-specific CTL (51), immune pressure may be exerted by CD8 T cells on viruses produced by both cell subsets. We propose that differences in peptidase activities among HIV-permissive cell types will be reflected in the mode of CTL-driven immune escape observed in HIV-1 sequences derived from patients. The combined effects of HLA-restricted mutations (impaired epitope processing or reduced binding to MHC) will tend to impair the ability of CTL to recognize infected cells. A comparison of early mutations occurring within the first year of infection with mutations occurring later during chronic infection in this large cohort of HIV-infected persons (using Gag, Pol, and Nef sequences) show that this mechanism is operative during both acute and chronic phases of the infection (data not shown). Future studies of mutational escape in other HIV genes will be especially interesting in light of these results.

The identification of motifs leading to immune escape is critical to a better understanding of immune failure in chronic infection and to the identification of immune responses with strong antiviral potential. Immune responses driving Ag processing mutations may reduce viral fitness to variable extents (9, 52), although the fold-reduction in in vitro viral fitness required substantially to reduce viral replication in vivo remains to be determined. Additionally, the degradation of mutated peptides may lead to the production of novel HIV epitopes and possibly to new CTL responses (53). Understanding and predicting how the virus and CD8 T cell responses evolve over time is critical to the development of novel vaccine strategies relying on the selection of various HIV sequences rather than HIV proteins of consensus sequences. It has been proposed to design immunogens that would elicit immune responses against both a consensus Ag and against the most common variants predicted to appear in vivo thereafter, pushing the virus to evolve toward unift variants (54). Identification of sequence signatures leading to impaired epitope presentation—and distinguishing them from irrelevant mutations linked to fitness and antiviral drug resistance—will help to predict patterns of viral immune escape at the population level. In addition, recent HIV vaccine design strategies explore the use of conserved areas of the virus as potential T cell-based immunogens (55–57). These fragments may need to be assembled—possibly with linkers—into a synthetic cassette encoding a protein that should remain immu- nogenic. The identification of flanking and intraepitopic motifs
rolling the efficiency of production and presentation of HIV epitopes to CD8 T cells provides a novel way to optimize peptide production from immunogens.

Disclosures

J.M.C. is an employee and shareholder of Microsoft. The other authors have no financial conflicts of interest.

References


Supplementary figure 1: The processing of B57-KF11 and B57-ISW9 epitopes is proteasome and aminopeptidase-dependent.
Supplementary figure 2: Validation of the mass spectrometry detection and analysis of peptide degradation

A

KF11 KAFSPEVIPMF

FF9 FSPEVIPMF

R=0.9988
p<0.0001

R=0.9805
p=0.0098

Peptide amount (fentomol)

Peaks surface

% total peptides

Repeated Injection time (min)

B

3-KF11 VEE-KAFSPEVIPMF

10 minute degradation
Repeated injections

3-KF11

2-KF11

KF11

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Supplementary figure 3: Single flanking residues drive the amount and antigenicity of HIV peptides produced in PBMC cytosol

A

B
Supplementary figure 4: Degradation profiles of a N-extended peptide and with flanking and intraepitopic mutations

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**Supplementary figure 1: The processing of B57-KF11 and B57-ISW9 epitopes is proteasome- and aminopeptidase-dependent.**

ISW9 (ISPRTLNAW; aa 15-23) and KF11 (KAFSPEVIPMF; aa 30-40) are 2 HLA-B57 restricted epitopes located in HIV-1 p24. HLA-B57-positive cells were preincubated for 30 minutes with control DMSO (black), proteasome inhibitor (MG132 10uM; dark grey), aminopeptidase inhibitor (bestatin 60uM, light grey) or both inhibitors and transfected with RNA encoding WT p24. 20 hours later transfected cells were used as target cells in a chromium release assay with ISW9- or KF11-specific CTL clone. Average of 3 transfection experiments, each run in triplicates. Two-way ANOVA with Bonferroni posttests. * p<0.5, ** p<0.01, *** p<0.001

**Supplementary figure 2: Validation of the mass spectrometry detection and analysis of peptide degradation**

A. Increasing amounts of peptides KF11 (left) and FF9 (right) diluted in 80% water, 15% acetonitril, 5% trifluoroethanol were analyzed by mass spectrometry. The peptide sequence and area under peak were calculated with Proteome discover. B. 5-KF11-3 was degraded for 10 minutes in cytosolic extracts and peptides in the mix were purified, diluted as mentioned in A, separated by RP-HPLC, identified and analyzed by mass spectrometry (showing 3-KF11 (circles), 2-KF11 (squares), KF11 (triangles)). Injection was repeated every 200 minutes (10 repeats) and the relative % of each peptide present in the mix is indicated. Average ± SD 3KF11: 87.09% ± 1.34%; 2-KF11 5.18%± 0.29%; KF11: 2.02% ± 0.21%
Supplementary figure 3: Single flanking residues drive the amount and antigenicity of HIV peptides produced in PBMC cytosol

A. 3-extended KF11 peptides (KAFSPEVPMF; aa 30-40 in p24) with single mutations flanking KF11 (WT VEE or mutants L, M, F, K, I, S, H, D, T, V) were degraded for 1h in PBMC cytosol. Fold increase production of KF11 and the relative PBMC aminopeptidase-specific hydrolytic activity of each amino acid was correlated with Spearman test. B. Correlation between antigenicity of the 60 minutes degradation products measured by cytolysis assay from 3-extended KF11 (WT VEE or mutants L, M, F, K, I, T) and the relative PBMC aminopeptidase-specific hydrolytic activity of each amino acid. Spearman test.

Supplementary figure 4: Degradation profiles of a N-extended peptide and of sequences with flanking or intraepitopic mutations

Peptide corresponding to Gag p24 epitope HLA-B57-ISW9 with a 3-residue extension with WT sequence (left), HLA-B57-restricted mutant sequence A146P with flanking proline mutation (middle panel), or sequence with intraepitopic mutation I147L (right panel) were degraded in PBMC cytosol for 60 minutes. Peptides identified by mass spectrometry are identified in blue, optimal epitope in green, and epitopes extended by 1, 2 or 3 residues in pale green. The mutated residue are colored in red. Profiles representative of 4 degradation experiments in extracts from 4 different donors.