TAP-independent human histocompatibility complex-Cw1 antigen processing of an HIV envelope protein conserved peptide

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Individuals with nonfunctional transporters associated with antigen processing (TAP) complexes are not particularly susceptible to viral infections or neoplasms. Therefore, their immune system must be reasonably efficient, and the present, though reduced, cytolytic CD8\(^+\) αβ T subpopulation specific for TAP-independent antigens may be sufficient to establish an immune defense protecting against viral infections in these individuals. The objective of the present study was to identify TAP-independent ligands from HIV gp160 protein. An analysis and comparison of complex human histocompatibility complex (HLA)-bound peptide pools isolated from large quantities of healthy or HIV gp160-expressing human cells was performed using mass spectrometry and bioinformatics tools. A conserved TAP-independent HLA peptide ligand endogenously processed and presented in infected human cells was identified. This ligand originates from the envelope protein bound to the HLA-Cw1 class I molecule with high affinity. It was concluded that HLA class I peptides derived from a large fraction of the N-terminal HIV envelope protein could be presented even in the absence of the TAP complex.

The killing of infected cells by CD8\(^+\) cytolytic T lymphocytes requires previous proteolytic degradation of viral proteins [1]. This antigen processing generates short peptides that are translocated to the endoplasmic reticulum lumen by transporters associated with antigen processing (TAP), where they assemble with newly synthesized β2-microglobulin and human histocompatibility complex (HLA) class I heavy chain. Following the initial assumption that the multicatalytic and ubiquitous proteasome is the only protease proficient in fully generating peptide ligands for HLA class I molecule binding, several studies have identified a growing number of alternative pathways that also contribute to endogenous antigen processing (reviewed in [2,3]). Individuals with mutations in the TAP gene that generate nonfunctional TAP complexes have been described (reviewed in [4]). Individuals with this HLA class I deficiency may be asymptomatic for long periods. Because TAP-deficient patients are not particularly susceptible to viral infections or neoplasms, their immune systems must be reasonably efficient. These individuals have sufficient repertoires of antibodies, natural killer (NK) cells, and CD8\(^+\) γδ T cells, but a reduced cytolytic CD8\(^+\) αβ T subpopulation specific for TAP-independent antigens, which together contribute to an immune defense that protects against severe viral infections. In two classic studies, Siliciano and colleagues [5,6] identified two nested TAP-independent epitopes in the HIV gp160 protein: residues 31–40 and 37–46 restricted by HLA-B18 and HLA-A3 class I molecules, respectively. No subsequent studies have addressed the existence of new TAP-independent ligands in this protein. To expand on the work by Siliciano and colleagues, we conducted a comparative immunoprotemic analysis of HLA ligands isolated from large quantities of TAP-deficient untreated or HIV gp160-expressing human cells. In this report, we describe the identification of yet another TAP-independent, HLA-Cw1-restricted, naturally processed ligand from the HIV gp160 protein.

HLA-bound peptides were isolated from \(4 \times 10^{10}\) healthy or recombinant vaccinia virus vSC25-infected T2-B27 transfectant cells as previously described [7]. T2, a line of TAP-deficient human cells that express HLA-A2, HLA-B51, HLA-Cw1, and HLA-E class I molecules on their surface [8], was transfected with HLA-B27 (a gift from Dr. David Yu, University of California, Los Angeles, California, USA). The vaccinia vector vSC25 encodes the envelope (ENV) glycoprotein gp160 from the HIV-1 strain IIIB [9] inserted in the genome of the western reserve strain. HLA-peptide complexes were isolated via affinity chromatography of the soluble fraction of cell extracts with the following monoclonal antibodies (mAbs) used sequentially: PA2.1 (anti-HLA-A2) [10], ME1 (anti-HLA-B27) [11], and W6/32 (specific for a nonhomomorphic HLA class I determinant) [12].

HLA class I peptides immunoprecipitated with each HLA-specific mAb were analyzed in three high-pressure liquid chromatography (HPLC) runs by micro liquid chromatography-mass spectrometry (μLC-MS/MS) using an Orbitrap XL mass spectrometer (Thermo-Fisher, San Jose, California, USA) [7]. Bioworks Browser 3.3.1 SP1 (Thermo-Fisher) was used for peak-list generation of the μLC-MS/MS data, and the HLA peptides were identified using the Sequest software tool and the human and virus parts of the NCBI database (January 2009), which includes 656,486 proteins. Identified peptides were selected if the following criteria were met: Sequest Xcorr more than 1.4 for singly, more...
than 2.2 for doubly, and more than 2.9 for triply charged peptides; P(\text{pep}) less than $10^{-3}$, and mass accuracy of 0.005 Da [7]. The purpose of the filtering criteria was to identify candidate HIV gp160 peptide MS/MS scans for further manual inspection to determine whether the MS/MS fragment ion fingerprint matched the identified peptide sequence. In addition, the corresponding synthetic peptide was made, and its MS/MS spectrum was used to confirm the assigned sequence.

The following synthetic peptides were used as controls in HLA/peptide complex stability assays: KPNA2 (GLVPFLVSV, HLA-A2-restricted) [13], HBV HBc19–27 (LPSDFFPSV, HLA-B51-restricted) [14], CMV pp657–15 (RCEPMISVL, HLA-Cw1-restricted) [15], HLA-A2 peptide leader (VMAPRTLVL, HLA-E-restricted), and C4CON (QYDDAVYLK, HLA-Cw4-restricted) [16]. The T2 line of TAP-deficient cells was used as previously described [17]. HLA expression levels were measured using the Abs monoclonal PA2.1 (anti-HLA-A2), monoclonal 3D12 (anti-HLA-E) [18], polyclonal H00003106-B01P (specific for HLA-B class I molecules; Abnova, Taipei, Taiwan), and polyclonal SC-19438 (specific for HLA-C class I molecules; Santa Cruz Biotechnology, Santa Cruz, California, USA) as previously described [17]. The fluorescence index was calculated as the ratio of the mean channel fluorescence of the sample to that of control cells incubated without the peptides. The binding of peptides was also expressed as EC$_{50}$, which is the molar concentration of the peptides producing 50% of the maximum fluorescence obtained at a concentration range between 0.001 and 100 μmol/L.

Sequential HLA-A2, HLA-B27, and a mix of HLA-B51, HLA-Cw1, and HLA-E-bound peptide pools were isolated from large quantities of either uninfected or vSC25-infected human TAP-deficient cells. These recovered peptide mixtures were subsequently separated by capillary reverse-phase HPLC and analyzed online by tandem mass spectrometry. In this analysis, two fragmentation spectra present in the vSC25-infected HLA-bound peptide pool that immunoprecipitated with the

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Fig. 1. Identification of the HIV ENV$_{57–65}$ ligand in infected cell extracts by mass spectrometry. The MS/MS fragmentation spectrum was obtained after ion trap mass spectrometry of the ion peak at m/z 1011.5 (upper left panel) or at m/z 506.2 (upper right panel) of the extract of vSC25-infected cells and the corresponding synthetic peptide at m/z +1 (lower left panel) or at m/z +2 (lower right panel). The vertical axis represents the relative abundance of the parental ion and each fragmentation ion detected. The horizontal axis corresponds to the m/z region in which significant daughter ions were detected. Ions generated in the fragmentation are detailed, and the sequence deduced from the indicated fragments is shown in the box in each respective panel. IF indicates internal fragments of peptide fragmentation. The difference between nominal and experimentally detected mono-isotopic ions was 0.001 and 0.003 Da for ion peaks at m/z 1011.5 and m/z 506.2, respectively.
W6/32 mAb but were absent in the control uninfected pool were identified at high confidence as peptides of the HIV ENV protein. Additionally, a human and viral proteome database search failed to reveal the identity of these spectra as human or vaccinia protein fragments, supporting the HIV viral origin of these sequences. The two different ion peaks at m/z 506.2 and 1011.5 corresponded to singly (Fig. 1, upper left panel) and doubly charged (Fig. 1, upper right panel) states of the peptide DAKAYDTEV, respectively. The DAKAYDTEV sequence is highly conserved between different HIV isolates (Supplementary Table 1, http://links.lww.com/QAD/A95). These peaks were assigned to the same viral amino acid sequence, which spans residues 57–65 of the HIV ENV protein. Virtually all significant fragments of both MS/MS spectra were assigned as daughter ions of the tentative peptide sequence (Fig. 1, upper panels). This theoretical assignment was confirmed on the basis of its identity with the MS/MS spectra of the corresponding synthetic peptide (Fig. 1, lower panels). No fragmentation spectra present in either HLA-A2-bound or HLA-B27-bound peptide pools were detected with sufficient confidence parameters as potential peptides of the HIV gp160 protein. Thus, these results indicate that a new TAP-independent ligand was endogenously processed and presented in the vSC25-infected cells.

Although the classic anchor motifs for HLA-A*0201 binding were described as Leu or Met at position 2 (P2) and aliphatic C-terminal residues (SYFPEITHI database, http://www.syfpeithi.de [19]), several HLA-A2-bound peptides previously described in the same database have Ala at P2 and Val C-terminal residues (for example, FASHVSPEV, EAAEVIILRV, KARDPHSGHFV, KACDPHSJGFV, AAGIGILTV), which is similar to the DAKAYDTEV ligand. HLA/peptide complex stability assays were performed to confirm that the sequential immunoprecipitation was performed correctly and to exclude the possibility of residual HLA-A2-bound DAKAYDTEV ligand that was not fully immunoprecipitated with the PA2.1 (anti-HLA-A2) Ab in the first round and immunoprecipitated in the third round with the W6/32 Ab (specific for a monomorphic HLA class I determinant). Figure 1a shows that, in contrast to the control HLA-A2 ligand, the KPNA2 peptide, induction of HLA-A2 complexes with the HIV ENV\textsubscript{57–65} peptide was not detected. Thus, this viral ligand does not bind to HLA-A2. The T2 human cell line also expresses HLA-B51, HLA-Cw1, and HLA-E class I molecules [8]. Therefore, to identify the HLA restriction of this ligand, new HLA/peptide complex stability assays using TAP-deficient T2 cells with specific anti-HLA-B, HLA-C, or HLA-E Abs were performed. No HLA stabilization was

![Fig. 2. Human histocompatibility complex stabilization assay with the HIV ENV\textsubscript{57–65} synthetic peptide ligand.](image)

The stability of human histocompatibility complex-A2 (HLA-A2) (a), HLA-B51 (b), HLA-Cw1 (c), and HLA-E (d) at the cell surface of T2 TAP-deficient cells was measured by flow cytometry. The indicated peptides were used at 200 μmol/l. The monoclonal antibodies (mAbs) used were monoclonal PA2.1 [anti-HLA-A2, (a)], polyclonal H00003106-B01P [anti-HLA-B class I molecules, (b)], polyclonal SC-19438 [anti-HLA-C class I molecules, (c)] and monoclonal 3D12 [anti-HLA-E, (d)]. Synthetic peptides HIV ENV\textsubscript{57–65} (circles), CMV pp65\textsubscript{7–15} (positive control, squares), and KPNA2 (negative control, single line) were titrated on cells expressing HLA-Cw1 (e), and stabilization of HLA class I molecules was measured by flow cytometry with the polyclonal SC-19438 Ab as in a–d. The results, calculated as fluorescence index (a–d) or EC\textsubscript{50} (e) values ± SD, are the means of four to five independent experiments. **Significant P values (P < 0.0001).
detected using either anti-HLA-B (Fig. 2b) or anti-HLA-E (Fig. 2d) Abs, indicating that the DAKAYDTEV peptide is not restricted by HLA-B51 or HLA-E class I molecules. In contrast, the numbers of HLA-peptide surface complexes induced by HIV ENV 57–65 synthetic peptide were similar to those induced by a well known HLA-Cw1 ligand, CMV pp85 7–15 (Fig. 2c), using the anti-HLA-C Ab. The consensus peptide binding motif for HLA-Cw1 is Ala or Leu at peptide position 2 [20]. Thus, the HIV ENV 57–65 nonamer is a natural HLA-Cw1 ligand.

Several studies have shown that peptides presented on TAP-deficient cell lines had decreased HLA binding affinity [21,22]. Thus, the relative HLA class I affinity of the DAKAYDTEV ligand was evaluated. This peptide bound to HLA-Cw1 in the range commonly found among other natural ligands. The HIV ENV 57–65 ligand efficiently stabilized HLA-Cw1 with an EC50 for HLA binding of 3±1 μmol/L, which is more efficient than the other optimal ligand, CMV pp65 7–15 (Fig. 2e).

A recent study defined different protease cleavage sites on HIV gp120 recognized by three major human proteases (cathepsins L, S, and D) important for antigen processing and presentation [23]. These or other uncharacterized proteases could be involved in the generation of both current HLA-Cw1 ligand and two previous TAP-independent epitopes identified in the HIV ENV protein [3,6]. These data support the hypothesis that the different cellular proteolytic systems contribute to the repertoire of presented peptides [2], thereby facilitating perhaps the immunosurveillance of infected individuals.

In summary, given that two nested TAP-independent epitopes (residues 31–40 and 37–46) were previously identified in the HIV ENV protein [3,6], the identification of yet another HLA ligand from this protein, the HLA-Cw1 ligand between residues 57 and 65, indicates that a large fraction of at least 65 residues of gp160 is processed by different endoproteolytic cleavages, resulting in the presentation by TAP-independent pathways in different HLA class I molecules.

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Interleukin-28B gene polymorphisms do not influence the susceptibility to HIV-infection or CD4 cell decline

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The critical role of interleukin-28B (IL28B)/interferon-α3 (IFN-λ3) polymorphisms on the susceptibility to hepatitis C virus infection and the response to peginterferon–ribavirin therapy has encouraged exploration of similar effects on other viruses. Given that IFN-α mediates anti-HIV-1 activity, the protective role of IL28B polymorphisms was examined in 29 seronegative individuals at risk for HIV-infection and in 68 HIV-positive carriers with and without rapid progression of immunodeficiency. No protective role of IL28B polymorphism was found examining both HIV-disease progression and HIV-protection.

Interleukin-28B (IL28B)/interferon-α3 (IFN-λ3) belongs to the IFN type III family, shares functional characteristics with IFN type I [1] and induces in-vitro protection against hepatitis C virus (HCV) infection [2]. Although several studies have demonstrated a role for single nucleotide polymorphisms (SNPs) nearby the IL28B gene on the spontaneous clearance [3,4], as well on the treatment-induced elimination [4–8] of HCV, it is currently unknown whether IL28B polymorphisms might similarly influence the outcome of other viral infections.

Although, in HIV-1, there is no evidence for spontaneous or treatment-induced cure, a subset of infected individuals, named long-term nonprogressors (LTNPs), demonstrate an intrinsic ability to control HIV-1 replication and defer immunodeficiency in the absence of antiretroviral therapy [9]. Moreover, evidence exists on another subset of individuals who despite being exposed to HIV remain persistently HIV-negative (exposed seronegative, ESN) [10]. A number of studies have been conducted in these special groups to ascertain whether potential immune mechanisms might account for their protection against HIV infection and/or disease progression [10,11]. Given the in-vitro antiviral effect of IFN-α on HIV replication [12], it is important to examine the role of IL28B polymorphisms on HIV infection.

Separate cross-sectional case–control studies were conducted in two different well characterized cohorts of patients. The first cohort ‘HIV disease progression’ included 68 individuals with chronic HIV infection in regular follow-up at Hospital Carlos III, Madrid. The second cohort ‘HIV protection’ included 29 HIV-serodiscordant sexual couples who attended Centro Sandoval, a sexually transmitted diseases clinic located in Madrid. To participate in the study, written informed consent for genetic testing was obtained from all individuals. The study protocol was evaluated and approved by the hospital ethics committee. Both plasma and peripheral blood mononuclear cells were drawn and stored for all patients.

IL28B rs12979860 SNP genotyping was conducted on DNA specimens using the 5’ nuclelease assay with allelespecific TaqMan probes (ABI TaqMan allelic discrimination kit, Applied Biosystems, Carlsbad, California, USA) and the ABI7900HT Sequence Detection System (Applied Biosystems) [13]. The main characteristics of the study population and the different parameters evaluated are expressed as median (interquartile range). Comparisons between groups were carried out using the χ²-test or Fisher’s exact test, as appropriate. All statistical analyses were performed using the SPSS software version 13 (SPSS Inc., Chicago, Illinois, USA). All P-values were two-tailed, and were considered as significant only when below 0.05.
The IL28B rs12979860 SNP is one of the most important discoveries of a genetic trait associated with control of a chronic viral infection. The fact that this SNP is located 3 kb upstream the IL28B/IFN-λ3 gene [5] and that IFN-λ exerts bioactivities that overlap those of type I IFNs, such as antiviral activity [1] and upregulates the intracellular expression of type I IFNs and APOBEC3G/3F, a well known anti-HIV-1 cellular factor [12]. Thus, it is somewhat unexpected that the IL28B SNP did not significantly influence HIV control, whereas the effect has shown to be quite strong on HCV infection. Therefore, large differences in the control of HCV and HIV infections may exist. The biological mechanisms involved in the association of the IL28B SNP over HIV control are still unclear. Although the CC genotype might modulate the regulation of IL28B protein expression, and in this way influence innate immunity, in spite of the previously reported IFN-λ-mediated anti-HIV-1 activity [12], we did not find any significant association between IL28B polymorphisms and HIV disease progression nor HIV protection. Thus, the biological mechanisms underlying the association of the IL28B SNP with HCV control could be other than the direct IL28B-mediated antiviral activity. Alternatively, different mechanisms might be operating in vivo in the control of HCV and HIV infections.

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### Table 1. Distribution of interleukin-28B rs12979860 genotypes in the study population.

| HIV disease progression cohort (n = 68) | LTNP (n = 30) | Typical progressions (n = 38) | OR (95% CI) (CC vs. CT/TT) | P |
|---------------------------------------|--------------|-----------------------------|---------------------------|---|
| CC                                    | 54% (16)     | 47% (18)                    | 1.27 (0.49–3.31)          | 0.63 |
| CT                                    | 33% (10)     | 45% (17)                    |                          |     |
| TT                                    | 13% (4)      | 8% (3)                      |                          |     |

| HIV protection cohort (n = 58)       |              |                             |                          |     |
|--------------------------------------|--------------|-----------------------------|---------------------------|---|
| CC                                    | 62% (18)     | 45% (13)                    | 2.01 (0.71–5.74)          | 0.19 |
| CT                                    | 35% (10)     | 38% (11)                    |                          |     |
| TT                                    | 3% (1)       | 17% (5)                     |                          |     |

CI, confidence interval; ESN, exposed seronegative; LTNP, long-term nonprogressor; OR, odds ratio; TP, typical progressor.
N.I.R., V.S., J.M.B., S.N., and J.McH. designed the study. N.I.R., C.R., J.M.B., and M.L. did the virological studies and collected the specimens. N.I.R., C.R., D.G. and J.McH. performed SNP genotyping. V.S. and J.R. were responsible for and analysed the demographics, clinical and therapeutic information of the study population. N.I.R., J.M.B., V.S., S.N. and J.McH. wrote the manuscript draft. All authors revised and approved the final submission.

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