TAP-INDEPENDENT HLA-Cw1 ANTIGEN PROCESSING OF AN HIV ENVELOPE PROTEIN CONSERVED PEPTIDE

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TAP-INDEPENDENT HLA-Cw1 ANTIGEN PROCESSING OF AN HIV ENVELOPE PROTEIN CONSERVED PEPTIDE

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Short title: TAP-independent HLA-Cw1 ligand

ABSTRACT

Background: Individuals with non-functional 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.

Objective: To identify TAP-independent ligands from HIV gp160 protein.

Methods: Analysis and comparison of complex HLA-bound peptide pools isolated from large quantities of healthy or HIV gp160-expressing human cells using mass spectrometry and bioinformatics tools.

Results: Identification of a conserved TAP-independent HLA peptide ligand endogenously processed and presented in infected human cells. This ligand originates from the envelope protein bound to the HLA-Cw1 class I molecule with high affinity.

Conclusion: 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.

INTRODUCTION

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 β_2-microglobulin and an HLA class I heavy chain. Following the initial assumption that the multi-catalytic 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 non-functional 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, 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's group identified two nested TAP-independent epitopes in the HIV gp160 protein: residues 31-40 and 37-46 restricted by HLA-B18 and -A3 class I

molecules, respectively [5;6]. 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 immunoproteomic 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.

METHODS

Isolation of HLA-bound peptides.

HLA-bound peptides were isolated from $4x10^{10}$ 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, -B51, -Cw1, and -E class I molecules on their surface [8], was transfected with HLA-B27 (a gift from Dr. David Yu, University of California, Los Angeles, CA). 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 (WR) strain. HLA-peptide complexes were isolated via affinity chromatography of the soluble fraction of cell extracts with the following mAbs used sequentially: PA2.1 (anti-HLA-A2) [10], ME1 (anti-HLA-B27) [11], and W6/32 (specific for a monomorphic HLA class I determinant) [12].

Electrospray mass spectrometry analysis.

HLA class I peptides immunoprecipitated with each HLA-specific mAb were analyzed in three HPLC runs by µLC-MS/MS using an Orbitrap XL mass spectrometer (Thermo-Fisher, San Jose, CA) [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 (Jan 2009), which includes 656,486 proteins. Identified peptides were selected if the following criteria were met: Sequest Xcorr >1.4 for singly, >2.2 for doubly, and >2.9 for triply charged peptides, P(pep) less than $1x10^{-3}$, 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.

HLA/peptide stability assays.

The following synthetic peptides were used as controls in HLA/peptide complex stability assays: KPNA2 (GLVPFLVSV, HLA-A2-restricted) [13], HBV HBc_{19-27} (LPSDFFPSV, HLA-B51-restricted) [14], CMV $pp65_{7-15}$ (RCPEMISVL, HLA-Cw1-restricted) [15], 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, CA) as previously described [17]. The fluorescence index (FI) 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 µM.

RESULTS AND DISCUSSION

A single HIV gp160 viral HLA ligand was differentially detected in vSC25-infected cells.

Sequential HLA-A2, -B27, and a mix of HLA-B51, -Cw1, and -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 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 I). These peaks were assigned to the same viral amino acid sequence, which spans residues 57-65 of the HIV envelope 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- or -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.

The identified HIV ENV_{57-65} is a canonical HLA-Cw1 ligand.

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: F**A**SHVSPE**V,** E**A**AEVILR**V,** K**A**RDPHSGHF**V,** K**A**CDPHSGHF**V,** A**A**GIGILT**V**), which is similar to the D**A**KAYDTE**V** 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_{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, -Cw1, and -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, -C, or -E Abs were performed. No HLA stabilization was detected using either anti-HLA-B (Fig. 3B) or -E (Fig. 3D) Abs, indicating that the DAKAYDTEV peptide is not restricted by HLA-B51 or -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. 3C), 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.

High binding affinity of the HIV ENV_{57-65} ligand to HLA-Cw1.

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 EC_{50} for HLA binding of 3 ± 1 µM, 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 both current HLA-Cw1 ligand and two previous TAP-independent epitopes identified in the HIV envelope protein [5;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 envelope protein [5;6], the identification here 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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Specific contributions to the published work:

- E.L., S.I., E.B., and I.B. performed research and analyzed data;
- R.G., F.L., and M.J. performed research;
- A.A. analyzed data;
- D.L. designed research, analyzed data, and wrote the paper.

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FIGURES

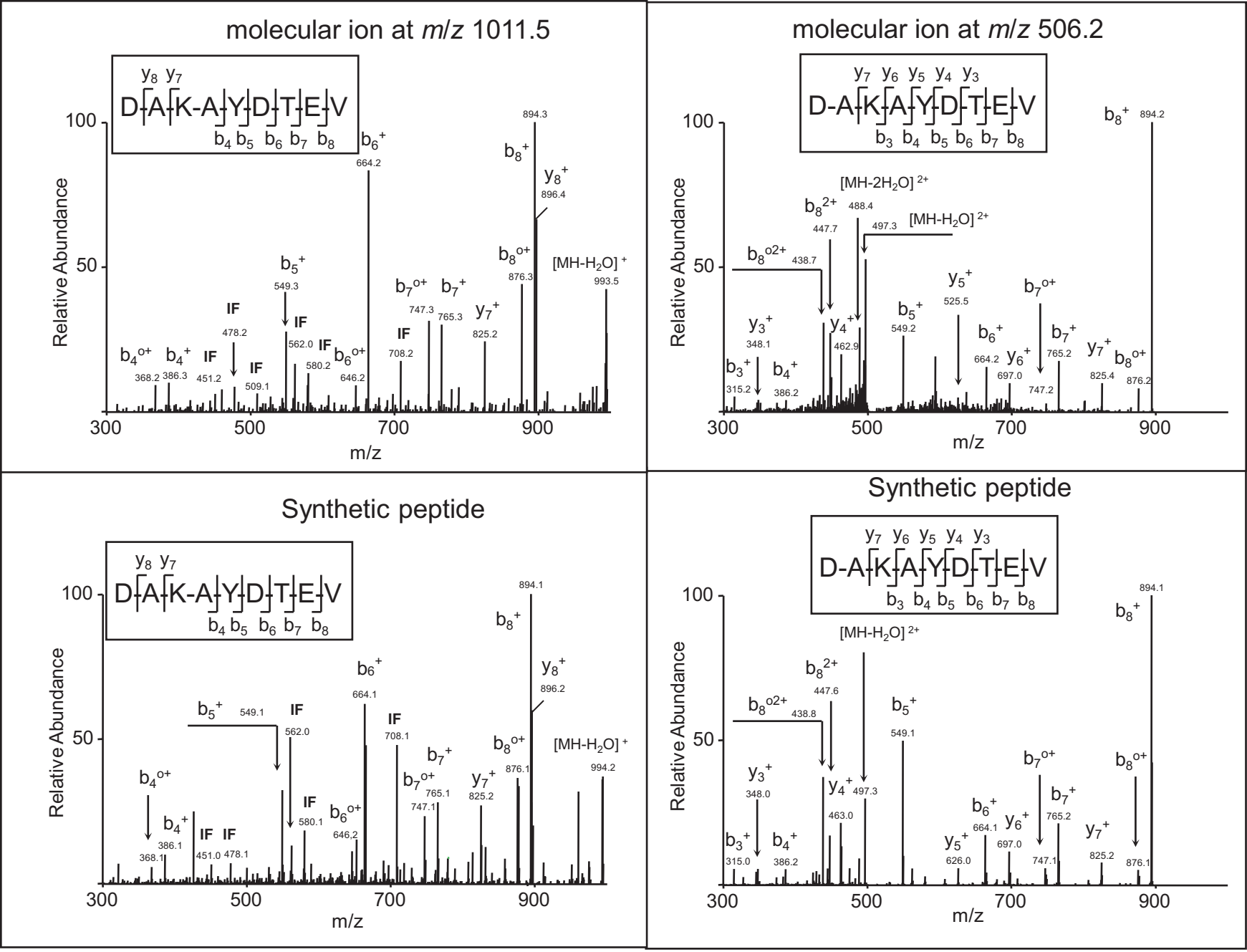
Figure 1. Identification of the HIV ENV$_{57-65}$ ligand in infected cell extracts by mass spectrometry.

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

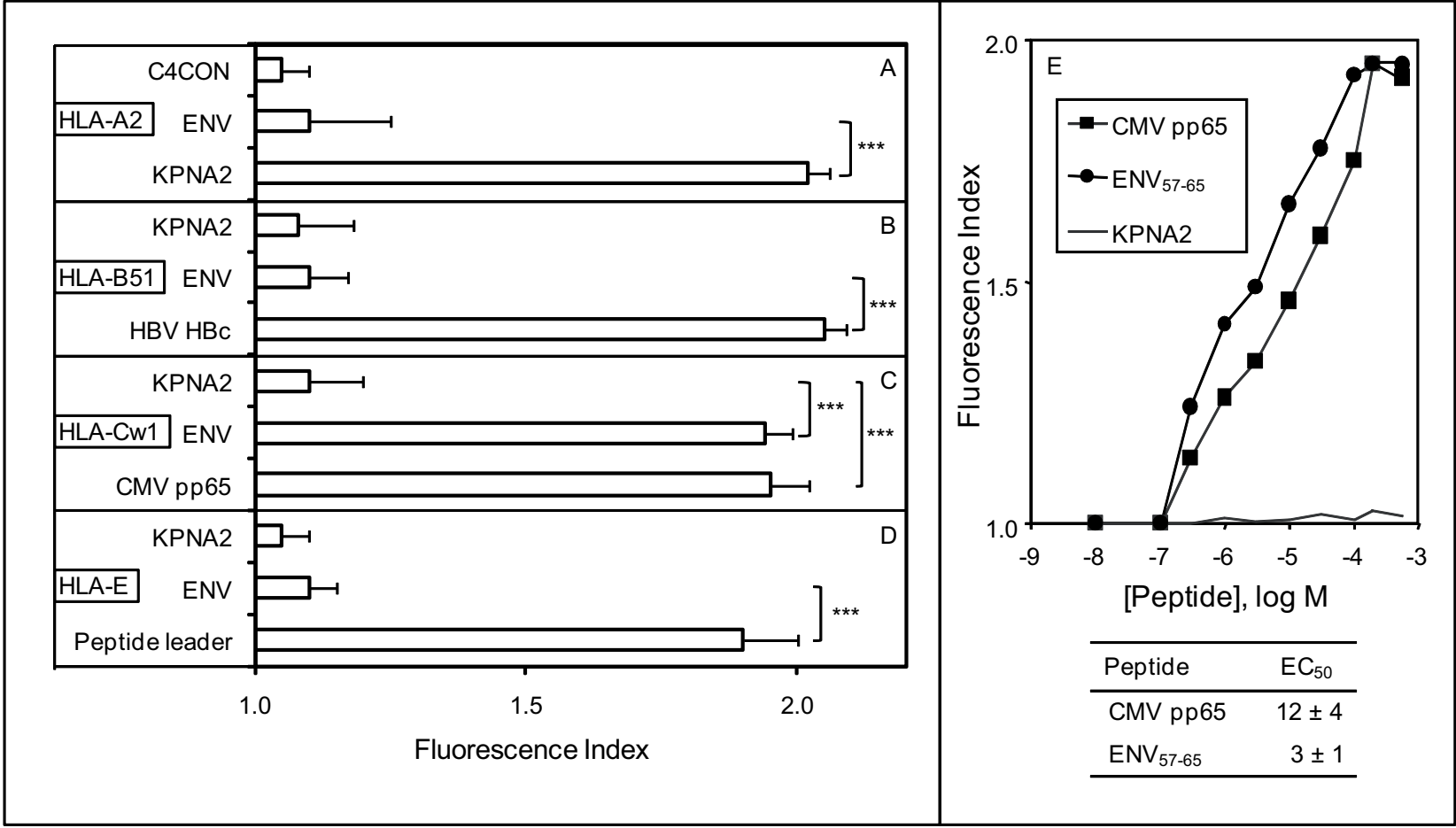
corresponding synthetic peptide at *m/z* +1 (lower left panel) or at *m/z* +2 (lower right panel). The vertical axis represents the relative abundance of the parental ion and each fragmentation ion detected. The horizontal axis corresponds to the *m/z* region in which significant daughter ions were detected. 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 monoisotopic ions was 0.001 and 0.003 Da for ion peaks at *m/z* 1011.5 and *m/z* 506.2, respectively.

Figure 2. HLA stabilization assay with the HIV ENV_{57-65} synthetic peptide ligand.

The stability of HLA-A2 (panel A), -B51 (panel B), -Cw1 (panel C) and -E (panel D) at the cell surface of T2 TAP-deficient cells was measured by flow cytometry. The indicated peptides were used at 200 µM. The mAbs used were monoclonal PA2.1 (anti-HLA-A2, panel A), polyclonal H00003106-B01P (anti-HLA-B class I molecules, panel B), polyclonal SC-19438 (anti-HLA-C class I molecules, panel C) and monoclonal 3D12 (anti-HLA-E, panel D). Panel E: synthetic peptides HIV ENV_{57-65} (circles), CMV $pp65_{7-15}$ (positive control, squares), and KPNA2 (negative control, single line) were titrated on cells expressing HLA-Cw1, and stabilization of HLA class I molecules was measured by flow cytometry with the polyclonal SC-19438 Ab as in panels A-D. The results, calculated as FI (panels A-D) or EC_{50} (panel E) values ± SD, are the means of 4-5 independent experiments. *** Significant P values ($p < 0.0001$).



Lorente et al. Figure 1



Peptide	EC_{50}
CMV pp65	12 ± 4
ENV_{57-65}	3 ± 1

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