

CUTTING EDGE

Cutting Edge: Class I Presentation of Host Peptides Following HIV Infection¹

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*Class I MHC molecules bind intracellular peptides for presentation to cytotoxic T lymphocytes. Identification of peptides presented by class I molecules during infection is therefore a priority for detecting and targeting intracellular pathogens. To understand which host-encoded peptides distinguish HIV-infected cells, we have developed a mass spectrometric approach to characterize HLA-B*0702 peptides unique to or up-regulated on infected T cells. In this study, we identify 15 host proteins that are differentially presented on infected human T cells. Peptides with increased expression on HIV-infected cells were derived from multiple categories of cellular proteins including RNA binding proteins and cell cycle regulatory proteins. Therefore, comprehensive analysis of the B*0702 peptide repertoire demonstrates that marked differences in host protein presentation occur after HIV infection. The Journal of Immunology, 2003, 171: 22–26.*

Major histocompatibility complex class I molecules exist as heterotrimers composed of a H chain, L chain, and peptide ligand (1). Class I molecules sample peptides from the proteome of the cell, transport the peptides to the surface, and interface with immune effectors where they communicate cellular fitness (2). This ability to sample and report on vast numbers of intracellular proteins has earned class I molecules the nickname “nature’s gene chips” (3).

Although it has been demonstrated that MHC molecules sample a vast array of endogenous proteins during the normal cellular lifecycle, characterization of host-protein-derived peptides after HIV infection has not been performed. Therefore, a fundamental question arises: what host-encoded peptides are uniquely presented on the surface of infected cells? Based upon the observation that HIV produces and interacts with multiple host-encoded proteins inside the cell (including Tsg101 (4) and

RNA polymerase II (5)), we hypothesized that host-protein-derived peptides are uniquely presented during infection.

We previously described a bioreactor-HLA-protein production method and a mass-spectrometric-ion-mapping system for comparatively screening class I-eluted peptide ligands (6, 7). In this study, we extend this approach to test the hypothesis that HIV infection alters the presentation of host-encoded peptides. Peptides eluted from HLA-B*0702 molecules produced in HIV-infected or uninfected cells were directly compared using mass spectrometry. Comparative mapping of HIV-infected and uninfected peptides results in the identification of 15 host-derived peptides uniquely presented on HIV-infected cells.

Materials and Methods

Soluble HLA production

Soluble HLA-B*0702 transfectants were produced as described (6) using Sup-T1 cells (7). Transfectants were cultured in a Unisyn CP2500 bioreactor unit (Biovest International, Minneapolis, MN) for 2 mo with continuous peptide collection. Approximately 30 mg of soluble HLA (sHLA)⁴ were collected from either uninfected or infected cells, supplemented with 1% Triton X-100, and stored at 4°C.

HIV infection

HIV-1 strain MN was propagated in Sup-T1 transfectants and monitored by p24 ELISA (Zeptomatrix, Buffalo, NY). For cell pharma infection, 3×10^9 cells were infected at a multiplicity of infection (MOI) of 0.5. For time course protein analysis, 1×10^9 cells were infected at an MOI of 4.5 for 2 h, washed once, and replaced in RPMI 1640 + 20% FBS.

Peptide purification

B*0702 molecules were affinity purified over a W6/32 affinity column. Peptides were eluted with 0.2 N acetic acid, brought to 10% acetic acid concentration, and heated to 78°C for 10 min. Fractions were purified in a stirred cell with a 3-kDa molecular mass cutoff cellulose membrane (Millipore, Bedford, MA). Peptides were reversed-phase-HPLC fractionated using a standard gradient of acetonitrile. Separate but identical peptide purifications were done from uninfected and infected cells.

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⁴ Abbreviations used in this paper: sHLA, soluble HLA; MOI, multiplicity of infection; PARP, poly(ADP-ribose) polymerase; MS, mass spectroscopy; HMG, high-mobility group protein; eIF, eukaryotic translation initiation factor; USP3, ubiquitin-specific protease 3; E6BP, E6-binding protein; HSP27, heat shock protein 27; TCP, tailless-complex protein; PTB, polypyrimidine tract-binding protein; Hu R, Hu Ag R.

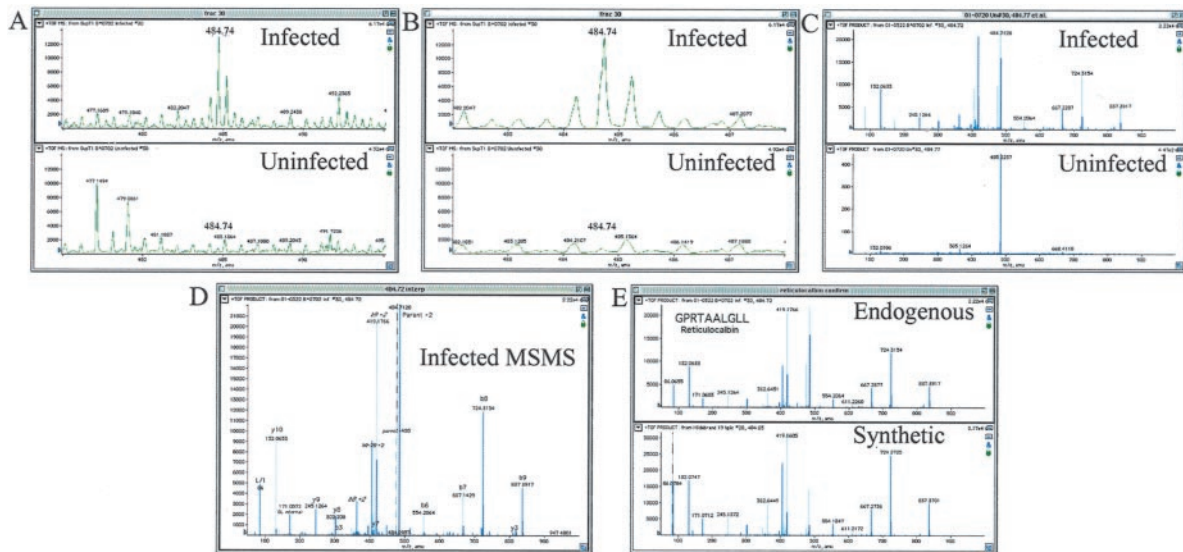


FIGURE 1. Mass spectrometric analysis of HLA-B*0702-bound peptides in HIV-infected cells. Peptides eluted from affinity purified sHLA were mapped on an ESI-QTOF mass spectrometer. Spectra were scanned for ion differences unique to infected cells. *A*, Initial scans were performed with windows of 200 atomic mass units and (*B*) subsequently zoomed to identify ion differences. *C*, Identified differences were subjected to MS/MS. *Top panel*, Peptide from uninfected cells; *bottom panel*, fragmentation of identical ion with identical atomic mass units in the uninfected fraction. Different fragmentation patterns indicated the absence of the peptide in the uninfected fraction. *D*, MS/MS sequence assignment from differentially expressed peptide. *E*, Synthetic peptides corresponding to putative sequence were subjected to MS/MS to verify sequence integrity.

Mass spectrometric analysis

Fractionated peptides were mapped by mass spectrometry as described (7). Peptides were nanoelectrosprayed (Protana, Odense, Denmark) into a Q-Star QTOF mass spectrometer (PerSeptive Sciex, Foster City, CA). Spectra from the same fraction in uninfected/infected cells were aligned to the same mass range and visually assessed for the presence of differences that were selected for manual and automated sequence assignment using the programs BioMultiview (PerSeptive Sciex) and MASCOT (Matrix Science, London, U.K.) (8). Synthetic peptides corresponding to each putative sequence were produced and subjected to mass spectroscopy MS/MS under identical collision conditions as the naturally occurring peptide and overlaid to confirm sequence identity.

Peptide binding assay

IC₅₀ values were determined using the HLA-B*0702 PolyScreen kit (Pure Protein, Oklahoma City, OK) according to the manufacturer's instructions. Fluorescently labeled control peptide and sHLA were incubated with each test peptide until equilibration of peptide replacement was reached as read on an Analyst AD plate reader (Molecular Devices, Sunnyvale, CA). IC₅₀ values were calculated using a dose-response curve.

Real-time PCR and Western blots

For Western blots, cells were lysed in electrophoresis buffer and total protein was quantified using the BCA Protein Analysis kit (Pierce, Rockford, IL). Proteins were transferred onto nitrocellulose membranes (Osmonics, Westborough, MA) before probing and detecting with commercially available Abs. For real-time PCR, total RNA was isolated using the Total RNA Isolation kit (Ambion, Austin, TX) and cDNA synthesized using the Retroscript kit. PCR was performed on a PE-7700 Light Cycler (Applied Biosystems, Foster City, CA) using primer pairs designed using Primer Express software. β -Actin was used as an internal standard. Relative transcript levels were calculated using the δ - δ cycle threshold method and normalized to zero in the uninfected cells.

Apoptosis analysis

Infected cells were treated with the Apo-Direct kit (BD Biosciences, Mountain View, CA) according to manufacturer's instructions and followed by analysis on a FACSCalibur. Poly(ADP-ribose) polymerase (PARP) cleavage was detected from the same Western blot lysates as above with an anti-PARP Ab (BD Biosciences).

Results and Discussion

Mass spectrometric mapping of peptides from infected and uninfected cells identifies 15 unique host-derived peptides

Following the harvest of B*0702/peptide complexes from both infected and uninfected cells, peptide ligands were eluted, fractionated, and each fraction was comparatively mapped using MS. Multiple peptides unique to HIV-infected cells were identified in these MS spectra (Fig. 1*A*). For example, comparison of spectra produced with peptides eluted from infected and uninfected cells identified a peak unique to the infected cells at 484.7 atomic mass units of fraction 16 (Fig. 1*B*). Peptide peaks unique to or up-regulated on HIV-infected cells were analyzed

Table I. IC₅₀ values for peptide binding to sHLA-B*0702

| Peptide Sequence | IC ₅₀ (nM) |
|--------------------------|-----------------------|
| TPQDLNITML ^a | 3047 |
| SPRTLNAWV ^a | 254.8 |
| AASKERSGVSL ^b | 317.3 |
| AARPATSTL | 209.8 |
| APAYSRAL | 214.7 |
| APKRPPSAF | 176.1 |
| GPRTAALGLL | 257.9 |
| IATVDSYVI | 218,900 |
| IPCLLLISFL | 5,980 |
| LPQANRDTL | 598.7 |
| LPTSHPKIV | 3,563 |
| NPNQKNVAL | 1,581 |
| QPRYPVNSV | 945.5 |
| RPYSNVSNL | 227.5 |
| SPNQARAQAL | 926.3 |
| STTAICATGL | 248,100 |
| TPQSNRPV ^m | 466 |

^a Control peptide, HIV-derived.

^b Control peptide, self-derived.

Table II. *HLA-B*0702 peptides differentially presented on infected cells*

| Peptide Sequence | Source Protein | Abbreviation | Main Cellular Function |
|-------------------------|--|--------------|--|
| APKRPPSAF | High mobility group protein 1/2 | HMG 1/2 | DNA binding |
| TPQSNRPV ^m a | RNA polymerase II, polypeptide A | RNA pol | Transcription |
| SPNQARAQAAL | Polypyrimidine tract-binding protein | PTB | mRNA processing and stability |
| NPNQKNVAL | Hu Ag R (ELAV-like 1) ^b | Hu R | mRNA processing and stability |
| AARPATSTL | Eukaryotic translation initiation factor 4GI | eIF4G | Translation |
| QPRYPVNSV | Tailless complex protein 1, α polypeptide | TCP-1 | Cytoplasmic protein chaperone |
| APAYSRAL | Heat shock protein 27 | HSP27 | Cytoplasmic protein chaperone |
| STTAICATGL | Ubiquitin-specific protease 3 | USP3 | Ubiquitin pathway |
| GPRTAALGLL | E6 binding protein (reticulocalbin 2) ^b | E6BP | Unknown; ubiquitin pathway? |
| IPCLLSFL | Cholinergic receptor, α 3 polypeptide | CholR | Signal transduction |
| RPYSNVSNL | Set-binding factor 1 | SBF-1 | Cell growth and differentiation |
| LPQANRDTL | MgcRacGap | MRG | Cell growth and differentiation |
| LPTSHPKIV | Suppressin | Sup | Cell cycle regulator |
| MAMMAALMA | Spark-like protein 1 (hevin) ^b | Spark-like | Antiadhesive extracellular matrix protein |
| IATVDSYVI | Tenascin C (hexabrachion) ^b | TenC | Similar to antiadhesive extracellular matrix protein |

^a Oxidized methionine.

^b Protein possessing more than one common name.

with tandem MS (Fig. 1, C and E). De novo sequence identification from MS/MS fragmentation patterns was performed on each peak unique to or up-regulated on infected cells (Fig. 1, D and E). Putatively identified ligands were analyzed for their predicted tandem MS fragmentation pattern (Fig. 1D) and compared with the spectra produced from uninfected cells (Fig. 1C). As a final confirmation of sequence integrity, peptides corresponding to the putative ligand sequences were synthesized and fragmented (Fig. 1E). Only peptides with identical experimental and control MS/MS fragmentation patterns were selected for further analyses.

As an independent verification of correct sequence assignment, we performed peptide-binding assays on each of the peptides identified through mass spectrometry. Synthetic peptides representing the peptide sequence were used in competition against a fluorescently labeled control peptide for binding to sHLA B*0702 molecules; IC₅₀ values were established for each peptide (Table I). All synthetic peptides bound to HLA-B*0702, most as strongly as the B*0702 control peptides. These data, combined with MS/MS fragmentation pattern analysis, confirmed that MS sequence analysis had yielded the correct ligand sequences.

Peptides presented at altered levels during HIV infection were derived from host proteins involved in multiple cellular pathways

Comparison of mass spectra from peptides eluted from infected and uninfected cells yielded 15 self protein-derived peptides showing altered expression on HIV-infected cells. These host-encoded peptides could be categorized by primary cellular function (Table II). Several of the peptides derived from proteins involved in RNA transcription or translation; peptides NPNQKNVAL and SPNQARAQAAL are both fragments of mRNA binding proteins, while the source protein for APKRPPSAF, high-mobility group protein (HMG) 1, facilitates the binding of transcription factors to DNA sequences (9). Another ligand in this category (AARPATSTL) derived from eukaryotic translation initiation factor (eIF) 4GI, a protein that plays a key role in cap-dependent mRNA translation initiation (10).

A second category of peptides unique to HIV-infected cells derived from cellular proteins involved in protein regulation. Peptides STTAICATGL and GPRTAALGLL derived from

ubiquitin-specific protease 3 (USP3) and E6-binding protein (E6BP), respectively. Protein USP3 hydrolyzes ubiquitin-protein bonds while E6BP can bind and alter the activity of a known ubiquitin ligase (11). Peptides APAYSRAL and QPRYPVNSV derived from source proteins heat shock protein 27 (HSP27) and tailless-complex protein (TCP)-1 (Table II) and participate in protein folding as cytoplasmic chaperones (12, 13).

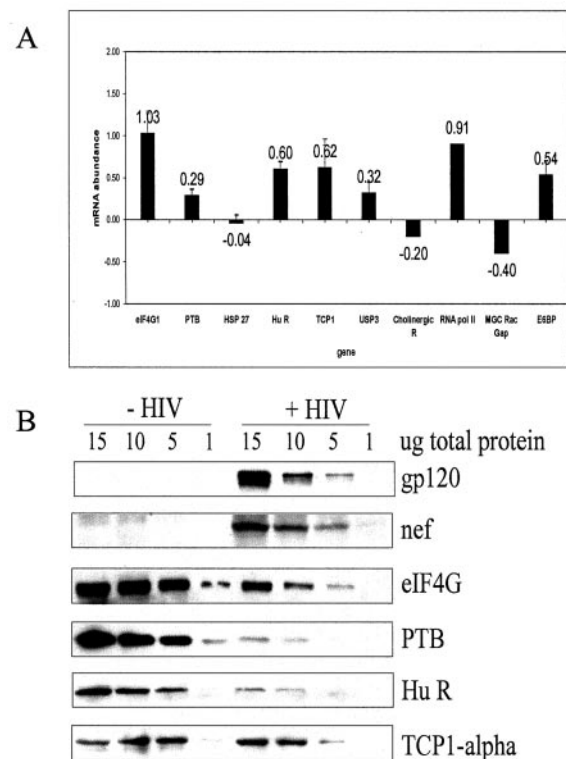


FIGURE 2. Transcriptional and protein levels of peptide-source proteins during a mixed-HIV infection of sHLA-B*0702 transfectants. *A*, Relative transcript levels of peptide source proteins as determined by real-time PCR. *B*, Western blot analysis of peptide-source proteins before (–HIV) and after (+HIV) infection.

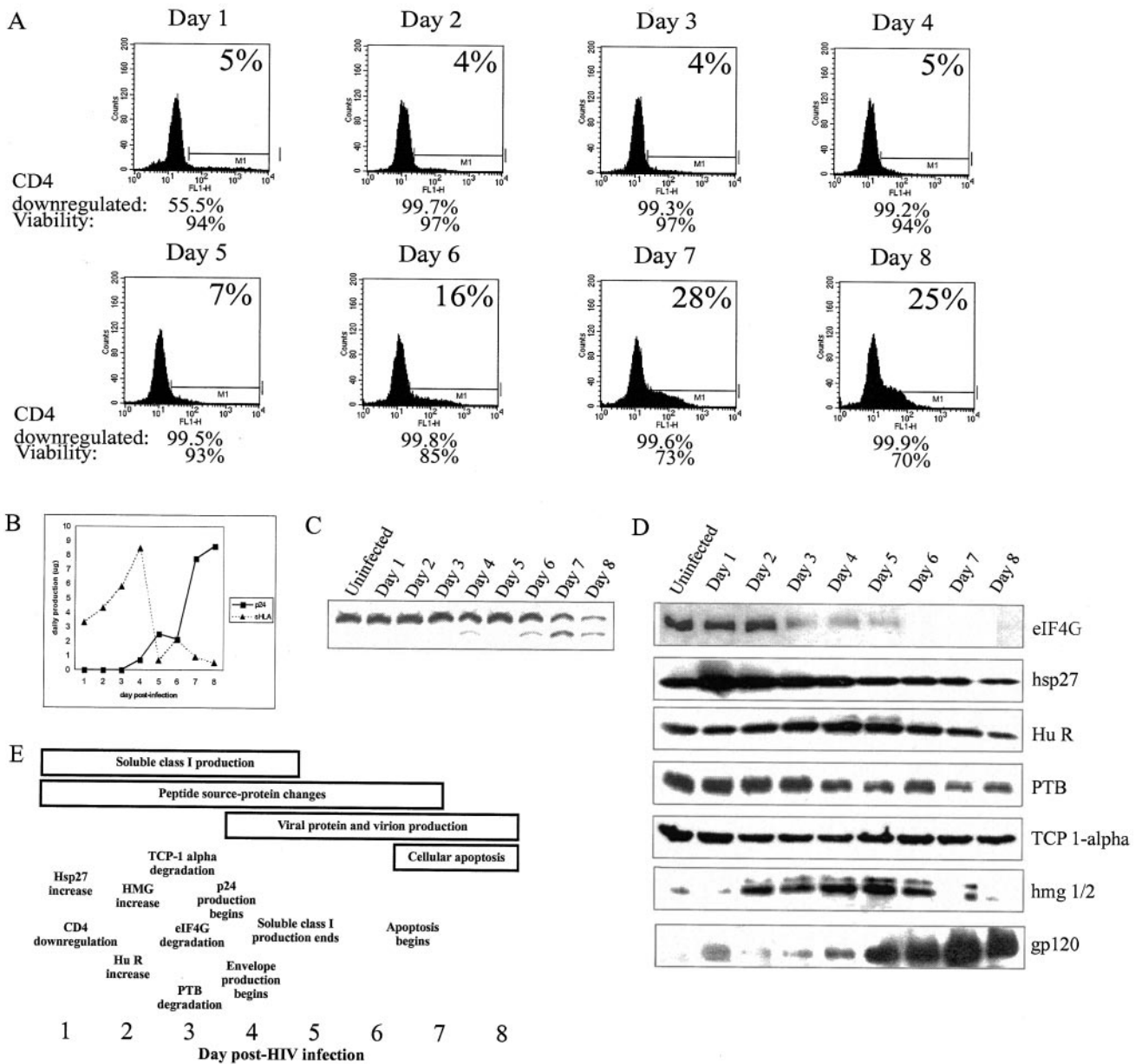


FIGURE 3. Kinetics of protein alterations during HIV infection of sHLA-B*0702 transfectants. Transfectants were infected with HIV MN and analyzed at 24-h intervals postinfection. *A*, Percentage of cells undergoing apoptosis and CD4 down-regulation during infection as determined by flow cytometry. Percent viability was determined by forward and side scatter. *B*, sHLA secretion and p24 secretion as measured by ELISA. *C*, Apoptosis analysis by Western blotting for PARP cleavage. *D*, Western blots of proteins representing unique peptides. *E*, Kinetics of protein changes during infection.

Unique peptides were derived from host proteins with altered protein levels during infection

Unique presentation of peptides during infection could be the result of multiple alterations inside the infected cell. Because HIV has been shown to transcriptionally up-regulate cellular genes (14) and gene overexpression can result in presentation by class I molecules (15), transcriptional up-regulation of genes representing unique peptides could be occurring before peptide presentation. To test this interpretation, real-time PCR was performed on mRNA transcripts from infected cells (Fig. 2*A*). Because the bioreactor system used for the harvest of sHLA proteins from infected cells represented a mixture of cells at different points in infection, T cells were infected with HIV and fed bi-weekly with uninfected cells. Once syncytia were evident visually, cells were pelleted, lysed, and real-time PCR was performed on ex-

tracted mRNA. Little change in the transcriptional level of the proteins examined was found. Thus, there was no pattern of transcriptional activation that would account for an overabundance of particular MHC-bound peptides on the infected cells.

A second possible mechanism for the presentation of unique peptides during infection was fluctuation in the levels of cellular proteins from which the peptides were derived. To examine this possibility, we performed Western blotting on total protein lysates from the same infected cells examined by real-time PCR. All of the proteins examined—eIF4GI, polypyrimidine tract-binding protein (PTB), Hu Ag R (Hu R), TCP-1, and HSP27—decreased in protein level in infected cells, indicating that degradation or turnover of the proteins was occurring (Fig. 2*B*). Thus, protein degradation during infection was the general mechanism for unique peptide presentation during infection.

Peptide changes occur early in infection

To determine the timing of protein changes, we performed a time course infection with HIV. Sup-T1 T cells were infected with HIV-1 MN at an MOI of 4.5 and cells and supernatants were sampled at 24-h intervals postinfection. Primary indicators of HIV infection were apparent early in the time course; almost 100% of cells exhibited down-regulation of CD4 by day 2 (Fig. 3A), while p24 release from infected cells began on day 4 (Fig. 3B). Flow cytometric measurement of cell viability and apoptosis (Fig. 3A) showed that a majority of the cells remained viable throughout the infection while TUNEL staining indicated that only one-fourth of the cells were undergoing apoptosis by day 8. As a secondary measurement of apoptosis, PARP cleavage was detected on day 7 (Fig. 3C). Interestingly, secretion of class I from infected cells precipitously dropped at day 5 (Fig. 3B), before the onset of apoptosis. These data indicate that cell death and apoptosis occur at late time points during infection of Sup-T1 T cells with HIV strain MN and that class I secretion predominates at early time points.

After establishing the kinetics of infection, Western blots were performed on the host proteins represented by overabundant peptides (Fig. 3D). Three of the proteins exhibited degradation by day 3 postinfection, including eIF4G, TCP-1 α , and PTB. Western blots further showed that HSP27, Hu R, and HMG 1/2 were up-regulated 1–2 days postinfection before dropping to lower levels later in the infection. The viral proteins envelope (gp120) and p24 (by ELISA) became apparent at day 4 of infection. As demonstrated in the mixed bioreactor infection, decreased levels of source proteins eIF4G, TCP-1 α , and PTB during HIV infection were not a result of decreased mRNA production (measured by real-time PCR at days 1–4, data not shown). Taken together, these data indicate that HLA-B*0702 presentation of unique peptides occurs as a result of protein changes occurring early in HIV infection (Fig. 3E).

In summary, the analysis of the HLA-B*0702 repertoire after HIV infection reveals a series of host-protein-derived peptides presented uniquely by infected cells. The host peptides most likely are presented as the result of protein level fluctuations that occur early during HIV infection. The consequence of these overabundant self peptides during infection is currently unknown but fits well into the paradigm of autoimmunity; autoimmune reactions are often present in individuals suffering from AIDS (16). Immune recognition of virus-induced host epitopes such as those reported in this study could function in the induction of autoimmune responses directly through increasing the concentration of self peptides on the cell surface.

Indeed, this mechanism is supported by a recent study demonstrating autoreactivity following measles virus-induced up-regulation of self peptides (17). Irrespective of possible function, these host-derived peptide ligands provide an expanded view of peptide presentation to the immune system following viral infection.

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