

The Human Immunodeficiency Virus Type 1 (HIV-1) Vpu Protein Interferes with an Early Step in the Biosynthesis of Major Histocompatibility Complex (MHC) Class I Molecules

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Summary

The human immunodeficiency virus type 1 (HIV-1) *vpu* gene encodes a small integral membrane phosphoprotein with two established functions: degradation of the viral coreceptor CD4 in the endoplasmic reticulum (ER) and augmentation of virus particle release from the plasma membrane of HIV-1-infected cells. We show here that Vpu is also largely responsible for the previously observed decrease in the expression of major histocompatibility complex (MHC) class I molecules on the surface of HIV-1-infected cells. Cells infected with HIV-1 isolates that fail to express Vpu, or that express genetically modified forms of Vpu that no longer induce CD4 degradation, exhibit little downregulation of MHC class I molecules. The effect of Vpu on class I biogenesis was analyzed in more detail using a Vpu-expressing recombinant vaccinia virus (VV). VV-expressed Vpu induces the rapid loss of newly synthesized endogenous or VV-expressed class I heavy chains in the ER, detectable either biochemically or by reduced cell surface expression. This effect is of similar rapidity and magnitude as the VV-expressed Vpu-induced degradation of CD4. Vpu had no discernible effects on cell surface expression of VV-expressed mouse CD54, demonstrating the selectivity of its effects on CD4 and class I heavy chains. VV-expressed Vpu does not detectably affect class I molecules that have been exported from the ER. The detrimental effects of Vpu on class I molecules could be distinguished from those caused by VV-expressed herpes virus protein ICP47, which acts by decreasing the supply of cytosolic peptides to class I molecules, indicating that Vpu functions in a distinct manner from ICP47. Based on these findings, we propose that Vpu-induced downregulation of class I molecules may be an important factor in the evolutionary selection of the HIV-1-specific *vpu* gene by contributing to the inability of CD8⁺ T cells to eradicate HIV-1 from infected individuals.

CD8⁺ T cells (T_{CD8+})¹ play a critical role in immune responses to many viruses. T_{CD8+} recognize MHC class I (MHC-I) molecules bearing viral peptides on the surface of virus-infected cells (1, 2). MHC-I molecules consist of three noncovalently associated subunits: an integral membrane glycoprotein of 44 kD (H chain), a small soluble protein (β₂-microglobulin [β₂m]), and an oligopeptide, usually 8–10 residues in length (3). Peptides are derived from a cytosolic pool of viral and cellular protein pre-

cursors (4, 5). Cytosolic peptides are transported into the endoplasmic reticulum (ER) by TAP (transporter-associated with antigen presentation [6–8]). TAP-transported peptides induce the release of newly synthesized H chain-β₂m tethered to TAP. The assembled tripartite complex reaches the cell surface via the standard exocytic pathway.

A number of viruses have evolved strategies to downregulate antigen presentation by MHC-I molecules (9, 10). Viral proteins may inhibit MHC-I gene promoter activity (11), retain class I molecules in the ER (12), destroy H chains in the ER (13, 14), dislocate nascent H chains into the proteasome pathway (15), or block the function of TAP (16–18). A decrease in class I expression on the cell surface occurs after infection with HIV type 1 (HIV-1; 19–21), and has been suggested as a reason for the inability of T_{CD8+} to eliminate the infection in vivo (22).

¹Abbreviations used in this paper: β₂m, β₂-microglobulin; CHAPS, 3-(3-cholamidopropyl)diethylammonio-1 propane sulfonate; ER, endoplasmic reticulum; pAbs-ex8, exon 8-specific polyclonal antibodies; rVV, recombinant VV; SIV, simian immunodeficiency virus; TAP, transporter associated with antigen presentation; T_{CD8+}, CD8⁺ T cells; VV, vaccinia virus.

The mechanism underlying this phenomenon is uncertain. Transcriptional analyses (11,19) provided evidence that this effect is due to a decrease in the transcription of genes encoding class I H chains. Howcraft et al. (11) used a swine MHC-I gene to demonstrate that the HIV transactivator Tat specifically decreases MHC-I gene promoter activity. However, Matsui et al. (23) found that Tat affects neither the expression, stability, nor transport of H chains. Consistent with these findings, we found that alterations in H chain-encoding transcripts do not account for decreases in MHC-I expression, and suggested that HIV affects class I expression at a posttranscriptional level (20, 21).

In the present communication, we examine the role of the HIV-1-specific Vpu protein in the downregulation of MHC-I molecules. Vpu is an 81-residue oligomeric type 1-anchored membrane protein that consists of a hydrophobic membrane anchor and a polar phosphorylated cytoplasmic tail (24–29). Among primate lentiviruses, Vpu is apparently encoded exclusively by HIV-1 and its close relatives (30). Like other so-called accessory genes of HIV-1, Vpu is not essential for virus replication *in vitro* (24, 25, 31, 32). However, Vpu consistently increases viral replication in T cell lines (24–26, 31, 32) and primary lymphocyte and macrophage cultures (33). It is possible that Vpu contributes to the increased virulence of HIV-1 relative to HIV-2 (34, 35). This hypothesis is supported by observations that Vpu enhances virus load and spread of infection in cynomolgus monkeys (36) and in SCID mice reconstituted with human immune cells (37).

Two distinct biological activities, which could contribute to the pathogenic potential of HIV-1, are attributed to Vpu: augmentation of virus particle release from the plasma membrane (24, 25, 30–33) and induction of proteolytic degradation of newly synthesized virus receptor CD4 in the ER (38, 39). One direct consequence of the Vpu-mediated CD4 proteolysis is to facilitate transport and processing of the envelope glycoprotein gp160 (38), which would otherwise be trapped in the ER because of the formation of stable complexes with CD4 (40). Therefore, in HIV-1-infected cells, the two separable functions of Vpu ensure that sufficient quantities of Env proteins reach the cell surface for incorporation into an increased number of budding virions.

In this report, we demonstrate that Vpu exhibits another important biological activity that could contribute to the increased pathogenicity of HIV-1 in comparison to HIV-2 (34, 35): Vpu decreases the cell surface expression of MHC-I molecules by interfering with an early step in class I biogenesis.

Materials and Methods

Cell Culture and Infection. T_{CD8+} cells were removed from buffy-coat cells of an HIV-negative HLA A-2,3-positive donor by magnetic beads coupled with an mAb specific for human CD8 (Dianova GmbH, Hamburg, Germany). The remaining CD8⁻ cells were stimulated with PHA (2 µg/ml) in the presence of 100 U/ml recombinant IL-2 for 2 d. 10⁶ stimulated cells were infected with 5 × 10⁴ tissue culture infectious doses, each of

HIV-1_{III}B (41, 42), HIV-2_{D205}, simian immunodeficiency virus (SIV)_{mac}, or HIV-1_{NL4-3} (43), and the isogenic *vpu* mutants HIVNL-U₃₅ (24) and HIVNL4-3/U_{2/6} (44). A3.01 cells (45) were cultivated in RPMI 1640/FCS. Routinely, 2 × 10⁶ reverse transcriptase units were used to infect 10⁷ A3.01 cells. After 15 h of absorption, cells were maintained at 10⁶ cells/ml as described previously (33, 46). Analyses of MHC-I expression was performed approximately at peak virus production, usually at day 12 after infection.

Flow Cytometry. 4 d after infection, aliquots from controls and infected PBL cultures were washed and incubated for 30 min on ice with mAb IOT 2 (Dianova GmbH). Cells were washed, incubated with FITC-conjugated goat anti-mouse Ig for 30 min on ice, washed, fixed in 3.5% formaldehyde, and analyzed on a cytofluorograph. To monitor infections, cells were fixed with 3.5% formaldehyde for 30 min at room temperature, washed, and treated for 20 min at room temperature with 0.25% Triton X-100 in PBS. Cells were then washed, incubated for 30 min at room temperature in 10% heat-inactivated sheep serum, washed, and incubated for 1 h at room temperature with purified polyclonal sheep antibody specific for HIV-1 p24^{gag} (Biochrom, Berlin, Germany). After extensive washing, cells were incubated with a 1:50 dilution of an FITC-conjugated polyclonal goat anti-sheep antibody and analyzed by flow cytometry. For detection of p27^{gag}, the FITC-conjugated mAb 2F12 (Biochrom) was used.

HeLa cells were infected for various times as indicated in the text with recombinant vaccinia viruses (rVVs) and incubated on ice for 30 min with FITC-conjugated antibodies specific for HLA class I molecules, W6/32 (Accurate Chem. & Sci. Corp., Westbury, NY), human β₂m (The Binding Site, Inc., San Diego, CA), mouse CD54 (PharMingen, San Diego, CA), mouse H-2 K^b, or human transferrin receptor (Becton Dickinson, San Jose, CA). Antibodies specific for mouse H-2 K^b were purified from the hybridoma cell line Y3 (American Type Culture Collection HB 176) and FITC-conjugated using standard procedures. After staining, HeLa cells were washed and resuspended in PBS supplemented with 10 µg/ml ethidium homodimer and analyzed by flow cytometry. Nonviable cells were excluded from analysis.

rVVs. rVVs were generated and propagated as described (47). Vpu-expressing rVVs were produced by cloning either wild-type Vpu from HIV-1_{NL4-3} (VV-Vpu; 43) or the mutant vpuDEL-1 (VV-U_{DEL1}; 31) behind the early/late VV p7.5 promoter. In brief, a 290-bp AflIII-KpnI fragment of the plasmid pSP-9 (24) or a 242-bp AflIII-KpnI fragment of the plasmid pSP-9/U_{DEL1} (46) were cloned into the NcoI-KpnI sites of plasmid pSC11 (48) yielding plasmids pSCVpu and pSC-U_{DEL1}, respectively. VV-ICP47 (49), VV-K^d (50), VV-K^b (50), and vCB-3 (51) have been described. VV-intracellular adhesion molecule (ICAM) expressing mouse CD54 was created as described (47) by inserting cDNA (52) behind the early/late VV p7.5 promoter into plasmid pSC11 (48).

Pulse Chase Metabolic Labeling. At day 4, p.i. aliquots of 10⁷ PBL were labeled with [³⁵S]methionine (2 mCi/ml) for 45 min, chased for the times indicated in the absence of radiolabeled methionine, and lysed in 90 µl of 3-(3-cholamidopropyl)diethylammonio-1 propanesulfonate (CHAPS) buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 100 mM NaCl, 0.5% CHAPS) + 15 µl CHAPS/deoxycholate buffer (CHAPS buffer with 2% deoxycholate). For the immunocollection of MHC-I molecules, lysates were precleared with mouse IgG (1.5 h, 4°C) coupled to protein A-Sepharose followed by specific immunocollection with HLA-specific mAb IOT2 (2 h, 4°C) coupled to protein A-Sepharose. Immunocollected proteins were separated in a 10% SDS-PAGE and analyzed by quantitative fluorography. HeLa cells infected at rVVs with 3–5

PFU/cell were starved for 20 min in methionine-free DMEM and labeled for up to 15 min with [³⁵S]methionine (2 mCi/ml). Cells were washed, chased in DMEM containing 1 mg/ml l-methionine, and lysed in CHAPS/deoxycholate buffer. Cell lysates were precleared by incubation at 4°C for 1 h with Gamma-Bind G-Sepharose preadsorbed with 20 μg of preimmune rabbit IgG, and incubated for 2 h with protein G-Sepharose preloaded with one of the following antibodies: mAb W6/32 (Harlan Sprague Dawley Inc., Indianapolis, IN), mAb SK81075 (Olympus Corp., Lake Success, NY), antihuman β₂m serum from rabbit (Dakopatts, Copenhagen, Denmark), mAb TW2.3 specific for vaccinia proteins E3L/E3L* (53), anti-Vpu sera from sheep (28) or rabbit (27), mAb 215 directed against conformed H chain K^d (54), polyclonal rabbit antibodies directed against exon 8 of H-2 K locus H chains (pAbs-ex8; reference 54), or anti-CD4 serum from rabbit (55). Immunoprecipitated proteins were separated in 10% or 12.5% acryl aide gels. Radioactive bands were visualized in fixed gels by fluorography and quantitated using a PhosphorImager.

Results

Effect of Vpu Expression on HIV-1-mediated Downregulation of Class I Molecules. We initially analyzed the potential contribution of Vpu to MHC class I cell surface downregulation by comparing the effects of infecting PHA/IL-2-activated, T_{CD8+}-depleted PBL with Vpu expressing HIV-1_{IIB} or the non-Vpu expressing viruses HIV-2_{D205}, or SIV_{mac} (Fig. 1 A). Surface expression of class I molecules was determined by cytofluorographic analysis of cells indirectly stained using a mAb specific for native, peptide-bearing class I molecules. As previously reported (19, 20), HIV-1 infection resulted in an ~10-fold decrease in the class I signal of a subpopulation of cells (Fig. 1 A d). This subpopulation correlates numerically with the number of cells expressing p24^{gag}, and double fluorescence confirmed that class I was selectively decreased in p24^{gag}-expressing cells (not shown). By contrast, infection with SIV or HIV-2 (Fig. 1 A, b and c) did not decrease class I expression to levels observed in HIV-1_{IIB}-infected cells despite the fact that a comparable percentage of cells expressed viral p24^{gag} antigens at similar levels (Fig. 1 A, f-h).

To specifically test the effect of Vpu on cell-surface class I molecule expression in the context of HIV infection, we used the molecular clone HIV-1_{NL4-3} (43) encoding either functionally characterized wild-type or mutant Vpus (24, 25, 31, 38, 39). T_{CD8+}-depleted, IL-2/PHA-activated PBL were infected with either wild-type HIVNL4-3 (43) or one of two isogenic mutant viruses. The first (HIVNL-U₃₅) encodes a 35 amino acid inactive Vpu protein with a COOH-terminal truncation (24). The second (HIVNL4-3/U_{2/6}) encodes a full-length mutant, Vpu_{2/6}, that cannot be phosphorylated due to exchanging Asn for phosphoacceptor Ser⁵² and Ser⁵⁶ residues (29). Vpu_{2/6} has lost the capacity to induce CD4 degradation, but maintains the ability to enhance virus secretion (44). Vpu_{2/6} exhibits similar structure, stability, oligomerization, membrane integration, and subcellular localization as wild-type Vpu (46, 56).

Parallel cultures of PBL were infected with equal infectious doses of the three viruses, and cell surface expression of

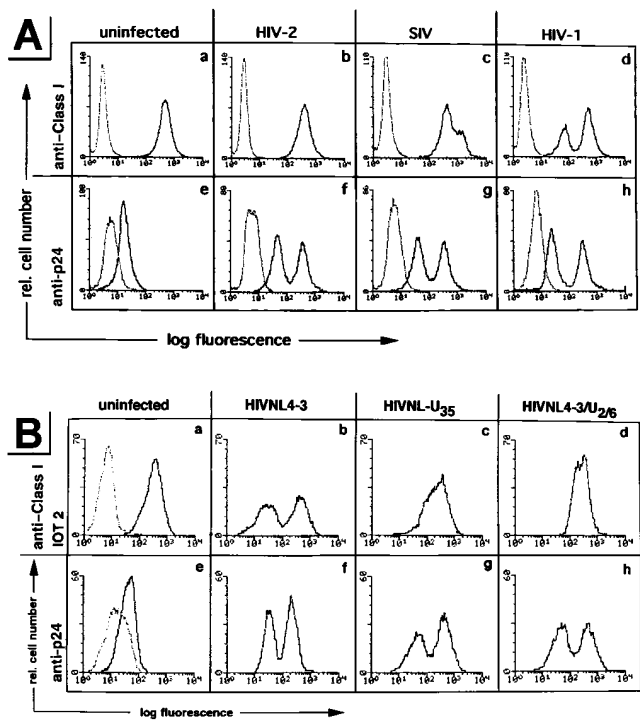


Figure 1. (A) Cell surface expression of MHC-I molecules is downregulated in T_{CD8+}-depleted and PHA/IL-2-activated PBL infected with HIV-1, but not in PBL infected with HIV-2 or SIV. Uninfected control (a); PBL infected with HIV-2_{D205} (b); SIV_{mac} (c); HIV-1_{BH10} (d); intracellular staining for viral core proteins p24/p27^{gag} (e-h). The thin curves to the left in each histogram represent negative controls. (B) Downregulation of MHC-I expression on HIV-1 PBL depends on the expression of biologically active Vpu protein. Uninfected controls (a, e, and i); T_{CD8+}-depleted and PHA/IL-2-activated PBL infected with wild-type HIVNL4-3 (b, f, and j), the vpu⁻ mutant HIVNL-U₃₅ (c, g, and k), and HIVNL4-3/U_{2/6} encoding nonphosphorylated Vpu_{2/6} (d, h, and l). The left curves in a, e, and i represent negative controls. Cells were stained with either conformation-specific mAb IOT2 (a-d). Virus expression was controlled by intracellular staining for p24^{gag} (e-f).

MHC-I molecules was analyzed 4 d after infection using a mAb that recognizes conformed class I molecules. Downregulation of surface class I molecules observed in the presence of wild-type Vpu (Fig. 1 B, b) was greatly compromised by either of the alterations in Vpu (Fig. 1 B, c and d). As above, the biphasic pattern of the histograms observed in the presence of Vpu reflects heterogeneity in HIV infection of the PBL cultures, as clearly revealed by cytofluorographic analysis of p24^{gag} expression (Fig. 1 B, f-h). Importantly, this demonstrated that there was no significant difference in either the number of p24^{gag}-expressing cells or the amount of p24^{gag} expressed by cells infected with the different clones. Thus, the inability of the Vpu mutants to decrease class I expression cannot be trivially attributed to impairment of viral replication and a concomitant decrease in the expression of other viral gene products. The residual slight decrease in class I expression exhibited by the Vpu mutants (Fig. 1 B, c and d) is consistent with recent reports that the HIV-1 accessory protein Nef induces endocytosis of cell surface MHC-I (57).

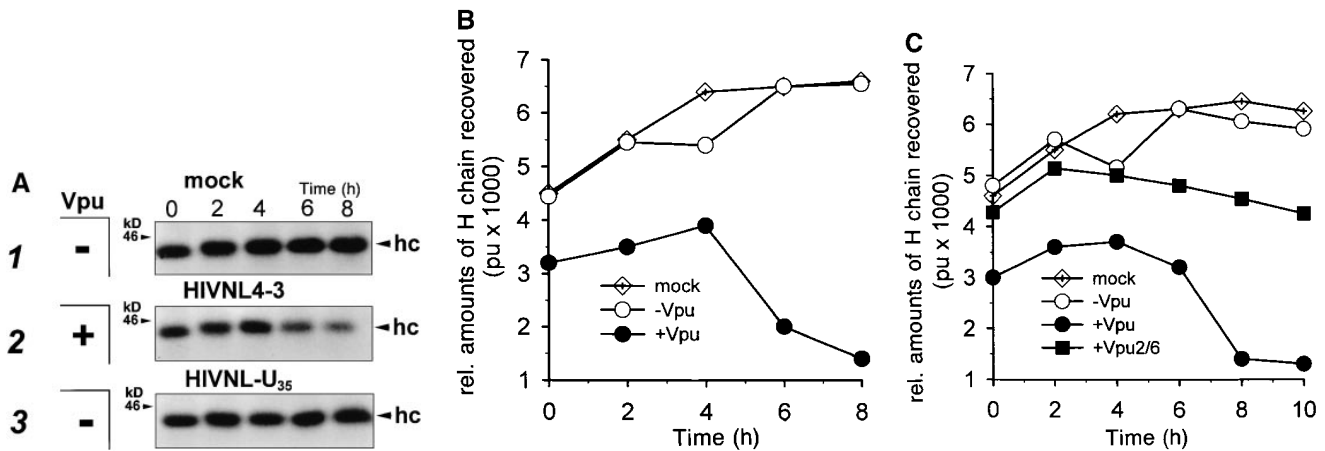


Figure 2. Vpu compromises generation of MHC class I complexes in HIV-1 infected PBL. (A) Pulse/chase analysis of MHC-I molecules in T_{CD8+} -depleted and PHA/IL-2-activated PBL infected with HIVNL4-3 (wild type, +Vpu), or the mutant HIVNL-U₃₅ (-Vpu); uninfected control (mock). MHC class I complexes were immunoprecipitated with mAb W6/32, separated in a 10% acryl aide gel, and analyzed by fluorography. Only parts of the fluorograms demonstrating H chain-specific bands in the range of 43 kD are shown. (B) Relative amounts of H chain proteins seen in (A) were quantitated by means of an image analyzer and blotted against the time of the chase period (pu, nonstandardized arbitrary PhosphorImager units). (C) T_{CD8+} -depleted and PHA/IL-2-activated PBL isolated from another donor were infected with HIVNL4-3 (+Vpu), HIVNL-U₃₅ (-Vpu), or HIVNL4-3/U_{2/6} expressing a nonphosphorylated mutant (+Vpu_{2/6}). Pulse/chase experiment, immunocollection, and SDS-PAGE analysis (not shown) were performed as described for Fig. 2, A and B. Relative amounts of H chains were quantitated with a PhosphorImager and plotted in arbitrary units (pu) against the time of the chase period.

Biochemical Characterization of Effects of HIV-1-encoded Vpu on Class I Biogenesis. To further characterize the effect of Vpu on the expression of MHC-I molecules, PHA/IL-2-activated, T_{CD8+} -depleted PBL from two donors were infected for 4 d with HIVNL4-3 or isogenic *vpu* mutants, and class I biogenesis was biochemically characterized (Fig. 2). After 45 min of radiolabeling with [³⁵S]methionine and chasing for various times at 37°C, class I molecules reactive with a mAb specific for conformed class I molecules were collected, separated by SDS-PAGE, and quantitated. Cells from either donor infected with wild-type virus demonstrated a 30–40% reduction in the amount of class I molecules recovered at the initiation of the chase, compared to uninfected or *vpu*-deficient cultures (Fig. 2, B and C). Additionally, an augmented decay of class I H chains was detectable in the presence of wild-type Vpu after a 4-h chase period (Fig. 2 A, 2) relative to either uninfected cells (Fig. 2 A, 1), cells infected with the Vpu-deficient mutant HIVNL-U₃₅ (Fig. 2 A, 3), or the Vpu phosphorylation mutant HIVNL4-3/U_{2/6} (Fig. 2 C). Note that the effects of Vpu on class I biosynthesis are underestimated in these experiments because not all cells are infected in each culture (Fig. 1 B, f–h). Consistent with the Vpu-dependent effects in PBL (Fig. 2), class I molecules were also affected in a Vpu-dependent manner in HIV-1-infected CD4⁺ T cell line A3.01 (not shown). Neither the amount of β_2m recovered from cell lysates nor its secretion into the culture supernatant was altered by Vpu in HIV-1-infected PBL or T cells (not shown), indicating that Vpu does not prevent class I assembly by reducing β_2m to limiting levels. Rather, we conclude that the Vpu-dependent downregulation of class I cell surface expression in cells infected with wild-type HIV-1 largely reflects Vpu-mediated instability of MHC-I

molecules due to their improper assembly or enhanced degradation.

Effects of rVV-expressed Vpu on Endogenous Class I Biogenesis. Several characteristics of HIV make it less than optimal as a means of characterizing the effect of Vpu on class I molecules. First, Vpu enhances the release of HIV from the plasma membrane of infected cells (24, 25, 31–33). Since HIV particles can contain large amounts of class I molecules (58), this was a possible confounding factor in the observed effect of Vpu on recovery of class I molecules (Fig. 2). Second, since HIV only infects a subset of cells, the effects of Vpu are obscured by processes unaffected in uninfected cells. To circumvent these problems, we produced VV-Vpu. As a control for the possible effects of Vpu mRNA, we also produced a rVV, VV-U_{DEL1}, expressing mRNA from the gene from the natural *vpu* mutant *vpu-DEL1* (31) that carries a 48-bp deletion within the Vpu transmembrane domain, resulting in the production of a 15-residue protein unrelated in sequence to Vpu (31).

Using a Vpu-specific antiserum, a protein with the predicted mobility of Vpu in SDS-PAGE was recovered from HeLa cells infected with VV-Vpu (Fig. 3 A), but not with VV-U_{DEL1} or from the uninfected culture. The stability of Vpu expressed by VV-Vpu was comparable to the half life of Vpu previously reported for HIV-1-infected or -transfected human cell lines (25, 44, 46). To characterize the biological activity of Vpu in VV-infected cells, we examined its effects on CD4 biogenesis. Mouse L929 cells were coinfected with VV-Vpu and vCB-3 (expressing wild-type human CD4; reference 51), radiolabeled for 7 min with [³⁵S]methionine, and then chased at 37°C for up to 240 min (Fig. 3 B). Detergent extracts were immunoprecipitated with an anti-CD4 antiserum, and analyzed by SDS-

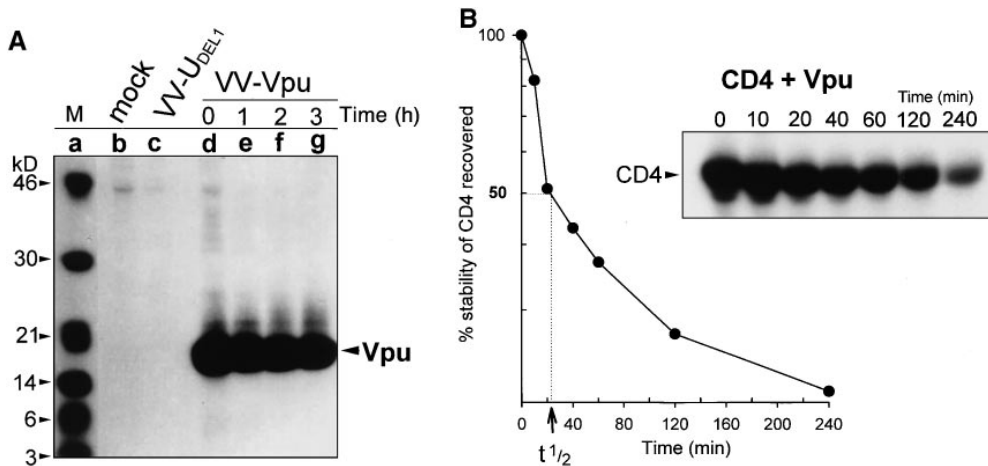


Figure 3. (A) Expression of Vpu by recombinant vaccinia viruses. HeLa cells were infected with rVVs expressing either wild-type Vpu (VV-Vpu) or the mutant Vpu_{DEL1} (VV-U_{DEL1}). 3 h after infection, HeLa cells were pulse labeled with [³⁵S]methionine for 25 min and cells expressing wild-type Vpu were chased for up to 3 h. Cell lysates were immunoprecipitated with anti-Vpu sera (sheep and rabbit), separated in a 12.5% acryl aide gel, and analyzed by fluorography. Positions of ¹⁴C-labeled molecular weight marker proteins are indicated on the left (M). (B) Mouse L929 cells were co-infected with rVVs expressing ei-

ther human CD4 (vCB-3) or wild-type Vpu (VV-Vpu). 3 h after infection, cells were pulse labeled with [³⁵S]methionine for 7 min and chased for up to 4 h. CD4 molecules were recovered with anti-CD4 serum, separated in a 10% acryl aide gel, and analyzed by fluorography. Only a part of the fluorogram demonstrating CD4-specific bands in the range of 55 kD is shown. Stability of CD4 present at different times during the chase period were calculated relative to the levels of CD4 present at the end of the pulse labeling (0), which was empirically defined as 100%.

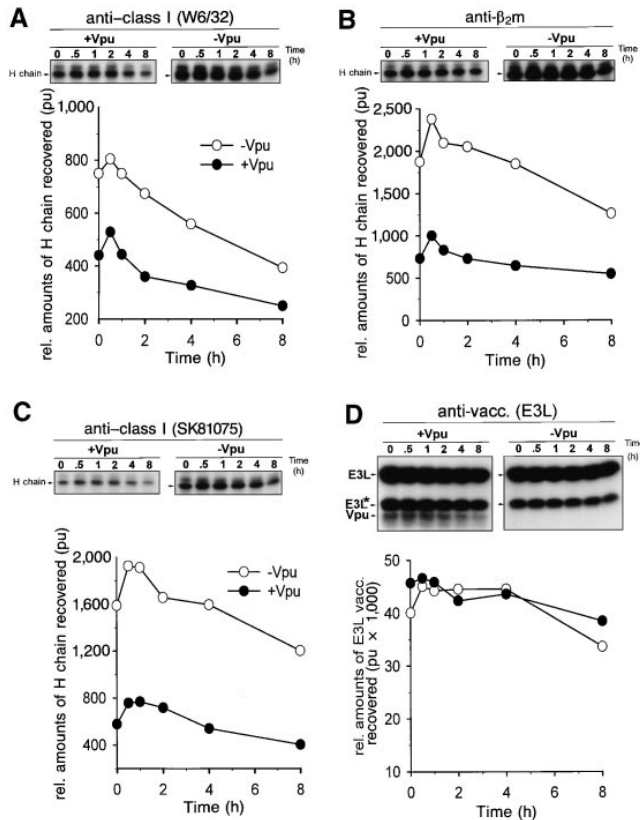


Figure 4. Vpu induces loss of MHC-I H chains in HeLa cells. Parallel cultures of HeLa cells were infected with rVVs VV-Vpu (+Vpu) or VV-U_{DEL1} (-Vpu), and 2.5 h after infection cells were pulse labeled with [³⁵S]methionine for 10 min and chased for up to 8 h. Aliquots of cell lysates were immunoprecipitated with mAb W6/32 (A), polyclonal antibodies specific for human β₂m (B), and mAb SK81075 specific for human H chains (C), separated in a 10% acryl aide gel, and analyzed by fluorography. Only bands corresponding to H chains in the range of 43 kD are demonstrated in the upper part; the quantitation of H chains by means of a PhosphorImager is demonstrated in the lower part. (D) Cell lysates were immunoprecipitated with antibodies specific for Vpu and the mAb TW2.3

PAGE (Fig. 3 B, inset). The kinetics of CD4 decay were determined by calculating the levels of CD4 present at different times relative to the levels of CD4 present at the end of the pulse (0 min), which was defined as 100% (Fig. 3 B). In the presence of rVV expressed Vpu, the half-life of CD4 was ~22 min. Similar Vpu activity was observed in CD4⁺ T cell lines and HeLa cells co-infected with VV-Vpu and vCB-3 (not shown). In the absence of Vpu, CD4 exhibited a $t_{1/2}$ ~4 h which is consistent with previously reported half-lives of CD4 in human cell lines (39, 46). Therefore, rVV-expressed Vpu has biological activity comparable to Vpu expressed in human cell lines transfected with vpu⁺ HIV-1 subgenomic expression vectors (38, 44, 46).

The effect of Vpu on MHC-I molecules endogenous to HeLa cells was examined 2.5 h after infection with VV-Vpu or VV-U_{DEL1} by radiolabeling cells for 10 min and chasing for up to 8 h. Class I molecules were collected from detergent lysates using either the mAb W6/32 specific for conformed class I molecules (Fig. 4 A), an antiserum specific for human β₂m (Fig. 4 B), or the mAb SK81075 specific for nonconformed human H chains (Fig. 4 C). Lysates were equalized to contain a comparable amount of incorporated [³⁵S]methionine. To control for possible differences between the infectivity of virus stocks, cell lysates were also subjected to immunocollection using a mixture consisting of polyclonal antibodies specific for Vpu and the mAb TW2.3 specific for vaccinia proteins E3L/E3L* (53; Fig. 4 D). Relative amounts of H chain and E3L proteins collected were quantitated by PhosphorImager analysis (Fig. 4, histograms). Expression of Vpu dramatically reduced the amount of class I H chains recovered with each of the

specific for vaccinia structural proteins E3L and E3L*. Proteins were separated in a 12.5% acryl aide gel. Parts of the fluorogram depicting a range between 14 and 30 kD are shown in the upper part; the quantitation of the VV E3L is shown in the lower part.

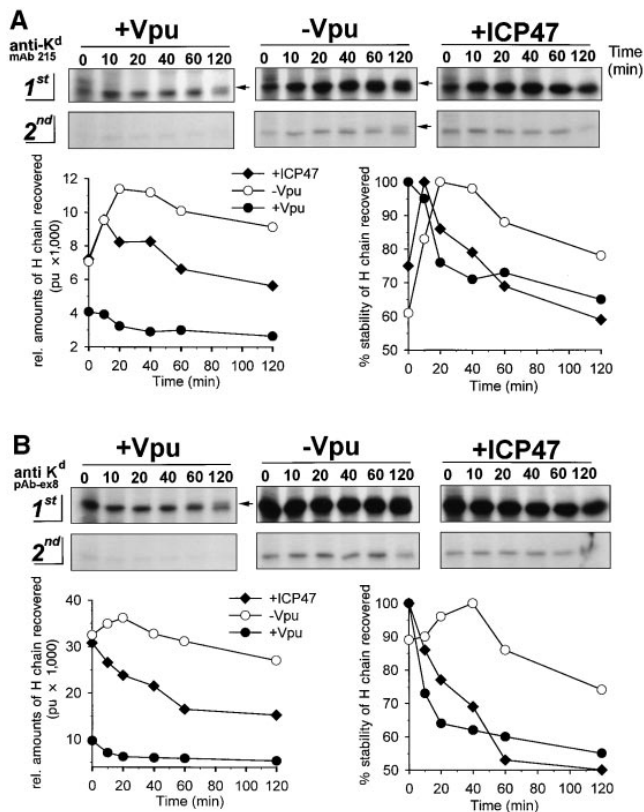


Figure 5. Vpu disturbs an early process in MHC-I biogenesis. Parallel cultures of HeLa cells were co-infected with VV-K^d expressing mouse H chain K^d together with VV-Vpu (+Vpu), VV-U_{DEL1} (-Vpu), or VV-ICP47 (+ICP47). 2.5 h after infection cells were pulse labeled with [³⁵S]methionine for 4 min, aliquoted in ice-cold medium, and chased at 37°C for up to 2 h. Half of the cell lysates were immunoprecipitated with mAb 215 (A) or with anti-K^d serum pAb-ex8 (B). Two rounds of immunocollection were conducted and H chain molecules collected were analyzed by SDS-PAGE followed by fluorography. Only bands corresponding to H chains are demonstrated in the upper part; the quantitation of H chains detected after sequential collection (1st and 2nd) by means of a PhosphorImager is demonstrated in the lower part, left histograms. Stability of H chains recovered is demonstrated in the right histograms. Arrows indicate mature glycosylated H chains detected after 2 h of chase period.

antibody preparations used at each of the time points tested (Fig. 4, A–C). This cannot be trivially attributed to differences between the infectivity of VV-Vpu and VV-U_{DEL1}, as shown by the nearly identical amounts of VV proteins E3L/E3L* recovered by mAb TW2.3 from each of the time points (Fig. 4 D). Consistent with our previous finding that Vpu does not affect the stability of the transferrin receptor (CD72) in HIV-1-infected macrophages (33), similar amounts of CD72 were recovered from VV-Vpu- and VV-U_{DEL1}-infected cells pulse radiolabeled for 10 min with [³⁵S]methionine and chased for up to 8 h (not shown).

Effects of rVV-expressed Vpu on rVV-expressed Class I Biogenesis. Notably, the effects of Vpu in class I biogenesis were evident immediately upon pulse labeling, indicating that Vpu affects early events in the assembly of class I molecules. To facilitate biochemical evaluation of Vpu effects on H chain synthesis, we examined the biosynthesis of mouse K^d

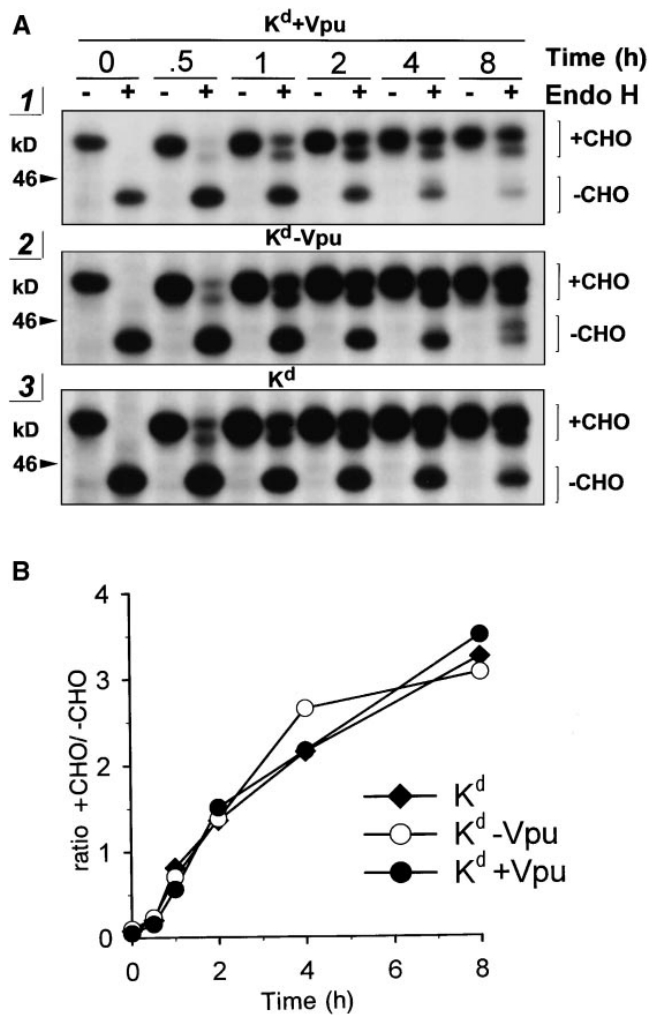


Figure 6. Transport of class I H chains is not affected by Vpu. Parallel cultures of HeLa cells were co-infected with VV-K^d together with VV-Vpu (K^d+Vpu) or VV-U_{DEL1} (K^d-Vpu), or VV-K^d alone (K^d). 2.5 h after infection, cells were pulse labeled with [³⁵S]methionine for 15 min and chased for up to 8 h. Class I molecules were immunocollected with a 1:1 mixture of mAb 215 and anti-K^d serum pAb-ex8. Bead-bound material was split and either not treated (-) or treated with endo H (+). Samples were analyzed on a 8% acryl aide gel. Parts of the fluorograms depicting H chain molecules are shown in A. The four bands in the treated samples represent H chains partially or completely resistant (+CHO) or sensitive (-CHO) to endo H treatment. Relative amounts of H chains seen in A were quantitated and the quotient +CHO/-CHO was calculated and plotted against the time of the chase period (B).

class I H chain expressed in HeLa cells co-infected with VV-K^d, VV-U_{DEL1}, or VV-Vpu (Fig. 5). The relatively high levels of K^d expression allowed for shorter labeling times with [³⁵S]methionine. Cells were radiolabeled for 4 min, and immediately placed on ice to stop cellular metabolism. Cells were then either maintained on ice or incubated for up to 2 h at 37°C. Cell lysates were equalized for [³⁵S]methionine incorporation, and class I molecules were collected with either mAb 215 specific for conformed K^d (Fig. 5 A, mAb 215; reference 54) or with rabbit serum specific for exon 8 (Fig. 5 B, pAb-ex8) that reacts with

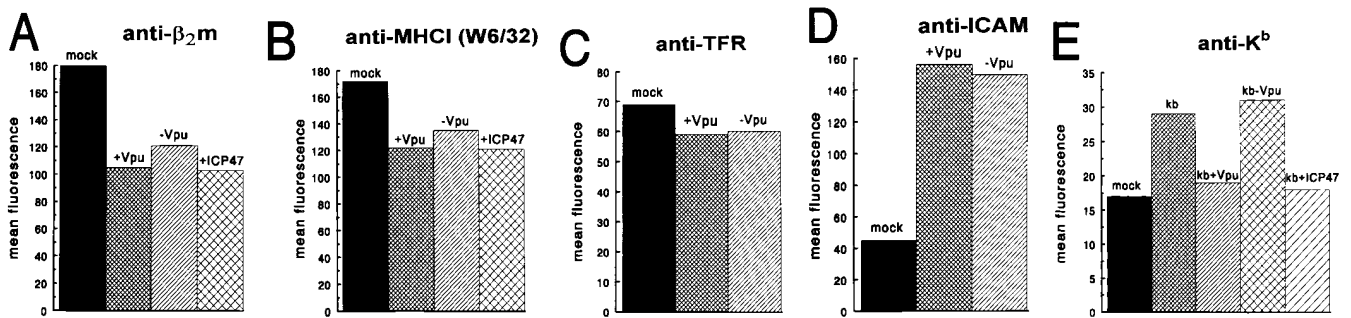


Figure 7. Vpu downregulates cell surface expression of MHC-I molecules. HeLa cells were infected with rVVs expressing either wild-type Vpu (+Vpu), the vpu mutant Vpu_{DEL1} (-Vpu), or the HSV protein ICP47 (+ICP47). In D and C cells were co-infected with rVV expressing the mouse glycoprotein ICAM-1 (CD54); in E cells were co-infected with rVV expressing mouse H chain K^b. Cells were stained 5 h after infection with antibodies specific for human β₂m (A), human MHC-I (mAb W6/32; B), human transferin receptor (TFR; CD72; C), mouse H chain K^b (E), or mouse ICAM-1 (D).

both folded and unfolded K^d molecules (54). To ensure complete recovery of H chains, supernatants from the first round of immunocollection were subjected to a second round of precipitation with the same Ab (Fig. 5, A and B, 1st and 2nd). The relative amounts of H chains recovered after sequential collection were determined by PhosphorImager analysis (Fig. 5, A and B, left histograms). In the absence of Vpu, the amount of K^d recovered by mAb 215 doubled over the first 20 min of chase, and then decayed slowly. This ~10 min half-life of K^d folding is consistent with previous findings regarding class I folding (3). We also detected a similar, but less marked, increase in K^d class I molecules collected by pAb-ex8. The increase in this case may be due to initial masking of exon 8 by interaction with molecular chaperones (60). We observed a number of remarkable effects of Vpu on K^d biogenesis. First, approximately two- to threefold less K^d reactive with either mAb 215 or pAb-ex8 was recovered from the unchased samples. Second, instead of an initial increase, the amount of K^d recovered precipitously declined over the first 20 min of chase period. Vpu had no significant effect on the stability of K^d that survived the initial effect, which are transported through the Golgi complex with kinetics similar to K^d synthesized in the control infected cells, as indicated by the recovery of a K^d form with lower mobility on SDS-PAGE (indicated by arrows in Fig. 5, A and B) that probably represents K^d with terminally sialylated N-linked oligosaccharides.

The fate of K^d that escaped the early action of Vpu was further examined in an experiment in which K^d H chains, collected from detergent lysates with a mixture of mAb 215 and pAb-ex8, was digested with endo H (Fig. 6). K^d possesses three N-linked oligosaccharides. Endo H removes high mannose N-linked oligosaccharides, but not N-linked oligosaccharides that have been acted on by Golgi mannosidase II. The rate of H chain transport to the mannosidase II-containing compartment was determined by calculating the ratio of H chain partially or completely resistant to endo H digestion (Fig. 6 A, +CHO) to endo H-sensitive H chains (Fig. 6 A, -CHO). Vpu expression had no significant effect on the kinetics of acquisition of endo H resistance (Fig. 6 B). This confirms that K^d molecules that

escape the early destructive effect by Vpu in the ER are exported and processed in a manner indistinguishable from H chains produced in cells not expressing Vpu.

Effects of rVV-expressed Vpu on Class I Cell-Surface Expression. The effects of Vpu on endogenous class I molecules present on the cell surface were investigated by testing rVV-infected HeLa cells for expression of conformed HLA molecules using either the mAb W6/32 or anti-β₂m antibodies directly conjugated to FITC (Fig. 7, A and B). Infection with rVV resulted in a 20–30% decrease in binding of either antibody, probably due to ongoing turnover of class I molecules combined with reduced replacement due to VV suppression on host cell protein synthesis. Staining was reduced a further 5–10% by Vpu, which is likely due to inhibition of expression of the reduced amounts of class I molecules produced in the face of the ongoing VV infection. This latter effect is selective for class I molecules since VV-Vpu and VV-U_{DEL1} infections reduced surface levels of endogenous CD72 nearly identically (Fig. 7 C), the reduction again probably related to VV induced suppression of host protein synthesis. Furthermore, Vpu had no effect on cell surface expression of mouse ICAM-1 (CD54) in HeLa cells co-infected with VV-Vpu and VV-ICAM (Fig. 7 D). In contrast, Vpu reduced cell-surface expression of K^b class I molecules co-synthesized in HeLa cells infected with VV-Vpu and VV-K^b (Fig. 7 E). These findings further demonstrate that Vpu selectively inhibits the cell surface expression of newly synthesized class I molecules, but has no or very minor effects on preexisting cell surface class I molecules.

Comparison of Vpu to HSV ICP47. Given the effect of Vpu on early events in class I biogenesis, it was of interest to compare the effects of Vpu with the ICP47 protein of HSV. ICP47 is a competitive inhibitor of TAP that greatly reduces the supply of class I-binding peptides (16–18, 61, 62). In several of the experiments described above, we included an additional sample in which VV-ICP47 was substituted for VV-Vpu. In the pulse chase experiments (Fig. 5, A and B, +ICP47), the effects of ICP47 and Vpu were quite disparate; In contrast to Vpu, ICP47 did not cause an immediate reduction in the amount of H chains recovered

at the end of the pulse-labeling time. In fact, the amount of H chains precipitated with the conformation-sensitive mAb 215 increased slightly over the first 10 min of chase period (Fig. 5 A, +ICP47). Consistent with previous findings (16–18, 61, 62), ICP47 expression results in the recovery of less conformed and total K^d over the chase period. Notably, the initial loss of immunoreactive class I induced by ICP47 occurs with a half-life approximately twice as long as the Vpu-induced loss (Fig. 5, A and B). Another important difference is that ICP47 delays the transport of class I molecules from the ER, as demonstrated by the failure of class I molecules to demonstrate the decrease in mobility associated with sialylation (Fig. 5, A and B, +ICP47). ICP47 and Vpu have a similar effect on class I surface expression (Fig. 7 E), blocking expression of VV-encoded K^b to the same extent as Vpu, while, like Vpu, having little effect on pre-existing surface HLA molecules (Fig. 7, A and B).

Discussion

As with many viruses, HIV-1 induces a rapid T_{CD8+} response in the host (63, 64). However, unlike most viruses, HIV-1 is not eradicated completely and continues to replicate for many years during the asymptomatic period of infection before the onset of AIDS. During this latent period, HIV-specific T_{CD8+} are active and present in PBL at very high levels relative to other viral infections (constituting up to 1% of circulating T_{CD8+}; reference 65), only gradually declining in parallel with T_{CD4+} during the progression to AIDS. The presence of a vigorous T_{CD8+} response raises the question of how the virus can persist in the face of an immune effector mechanism that is usually highly effective at eradicating virus. One possibility is that a subset of virus-producing cells are resistant to T_{CD8+}-mediated anti-viral effects.

Several mechanisms, including antigenic drift (63, 66, 67, 68), exhaustion of T_{CD8+} response by high level of antigen exposure (69), or deficiencies in HIV-1 antigen processing and presentation (11, 19–21, 57) have been proposed for resistance to T_{CD8+}-mediated lysis. In the present paper, we show that Vpu interferes with the expression of MHC-I molecules on the surface of HIV-1-infected cells. Indeed, viruses lacking Vpu demonstrate only a minor decrease in class I expression. It seems, therefore, that the Tat-mediated decrease in class I transcripts (11) plays little role (23) in the decrease in class I expression. Rather, we conclude that Vpu is required for most of the inhibition of cell surface MHC-I expression seen in HIV-infected cells, and propose that this may contribute to the inability of HIV-specific T_{CD8+} to eradicate the virus in infected individuals.

When expressed in the context of HIV-1 infection, we observed two effects of Vpu on class I molecules: (a) decreased recovery of class I H chains collected with immature N-linked oligosaccharides immediately after [³⁵S]methionine labeling and (b) decreased stability of class I molecules with mature N-linked oligosaccharides. Only the former effect was observed using rVV to express Vpu. There are

several explanations for this discrepancy. First, Vpu may act in concert with other viral gene products to affect the stability of class I molecules expressed on the cell surface. In this regard, it was recently reported that the HIV Nef gene product induces the degradation of cell surface class I molecules (57). Possibly, the activity of Nef and/or Vpu is increased by coexpression of both proteins. Second, alterations in cellular metabolism induced by rVV infection may interfere with the ability of Vpu to affect cell surface class I molecules. Third, Vpu is known to augment by two to threefold virus release from PBL (33). Since in HIV-1 particles, class I molecules constitute up to ~20% of the concentration of Gag protein on a molar basis (58), it is likely that at least some of Vpu-dependent loss in class I molecules with mature N-linked oligosaccharides is due to increased virion production with its attendant increase in class I shedding.

When expressed by rVV, Vpu reduced the amount of class I molecules recovered after brief labeling with [³⁵S]methionine, and, during a 20-min chase period, Vpu also decreased the metabolic stability of the H chains with immature N-linked oligosaccharides that escaped the immediate effects of Vpu. We interpret the latter phenomenon to represent the continuation of the same process resulting in decreased recovery of pulse-labeled heavy chains. Importantly, the effect of Vpu on class I molecules was clearly distinguished from that of ICP47 which acts by blocking the peptide transporting activity of TAP. This indicates that the effects of Vpu on class I biogenesis do not result from a decrease in the quantity or quality of peptides available for class I binding. We have not formally demonstrated that Vpu induces the degradation of MHC-I molecules. It is also possible that the decreased recovery of class I molecules is due to Vpu-induced decreases in solubility of class I molecules in the detergents used for extraction, or to the association with proteins that block antibody accessibility to class I molecules.

How might Vpu compromise biogenesis of class I molecules? It was recently demonstrated (46) that the two previously defined biological functions of Vpu, CD4 degradation and regulation of virus release, are controlled by two separable structural and functional domains. CD4 degradation requires phosphorylation of the Vpu cytoplasmic tail (44) and the binding of Vpu to CD4 (70) trapped in the ER by complex formation with the HIV-1 envelope precursor gp160 (40). In contrast, the effect of Vpu on virus particle release is regulated by an ion channel activity of its transmembrane domain (71, 72), occurs in a post-ER compartment (44), and does not require phosphorylation of Vpu (44). Vpu, therefore, possesses at least two distinct activities: first, alteration of the ionic milieu in a post-ER compartment, and second, targeting of proteins located in the ER for destruction. Our proposal that Vpu induces a rapid decay of nascent class I H chains is more consistent with the known ER activity of Vpu, and, based on the ability of Vpu to induce proteolysis of nascent CD4 in the ER (38, 39), it is likely that Vpu acts similarly on MHC class I molecules.

It was recently demonstrated that the US2 and US11 gene products of human cytomegalovirus are able to induce the rapid degradation of class I H chains (15, 73). Both act by routing newly synthesized class I molecules to the cytosol, where degradation requires the activity of proteasomes, in as much as it was blocked by proteasome inhibitors (15). The interaction of H chains with one of the components of the translocon suggest that US11 directs H chains in a retrograde fashion through the sec61-containing pore used to insert proteins into the ER (73). Indeed, based on this and other recent findings in *Saccharomyces cerevisiae* (74), it seems likely that many cases of presumed degradation of membrane proteins by ER proteases are, in fact, due to routing of proteins to the cytosol where they are degraded in a process requiring active proteasomes. It is necessary to reexamine if the Vpu-induced CD4 degradation involves the ubiquitin-proteasome pathway and whether a similar process is

used for class I molecules. It will also be of great interest to determine the shared features of class I H chains and CD4 that target them for Vpu-induced destruction while other glycoproteins such as Env, CD8 (75), CD54, and CD72 are spared.

In summary, our data indicate that Vpu is the major factor in the reduction of MHC class I molecules on HIV-1 infected cells. This adds another function to the two previously ascribed to Vpu, CD4 degradation and virus release. It is possible that one or more of these functions is an unintended byproduct of the other(s). However, it is equally plausible that each contributes to the evolutionary fitness of the virus. The relative contributions of these effects may vary depending on the nature of other viral genes or the host response to the virus. It will be a challenge to determine the biological significance of these three functions of Vpu.

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