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Nuclear Export of Vpr Is Required for Efficient Replication of Human Immunodeficiency Virus Type 1 in Tissue Macrophages

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Retroviruses must gain access to the host cell nucleus for subsequent replication and viral propagation. Human immunodeficiency virus type 1 (HIV-1) and other primate lentiviruses are distinguished from the gammaretroviruses by their ability to infect nondividing cells such as macrophages, an important viral reservoir in vivo. Rather than requiring nuclear membrane breakdown during cell division, the HIV-1 preintegration complex (PIC) enters the nucleus by traversing the central aqueous channel of the limiting nuclear pore complex. The HIV-1 PIC contains three nucleophilic proteins, matrix, integrase, and Vpr, all of which have been implicated in nuclear targeting. The mechanism by which Vpr can display such nucleophilic properties and yet also be available for incorporation into virions assembling at the plasma membrane is unresolved. We recently characterized Vpr as a nucleocytoplasmic shuttling protein that contains two novel nuclear import signals and an exportin-1-dependent nuclear export signal (NES). We now demonstrate that mutation of this NES impairs the incorporation of Vpr into newly formed virions. Furthermore, we find that the Vpr NES is required for efficient HIV replication in tissue macrophages present in human spleens and tonsils. These findings underscore how the nucleocytoplasmic shuttling of Vpr not only contributes to nuclear import of the HIV-1 PIC but also enables Vpr to be present in the cytoplasm for incorporation into virions, leading to enhancement of viral spread within nondividing tissue macrophages.

Human immunodeficiency virus type 1 (HIV-1) and other primate lentiviruses are able to infect nondividing cells, notably terminally differentiated macrophages (41), an important viral reservoir within the infected host (31, 34, 53). This biological feature distinguishes the lentiviruses from the oncoretroviruses (or gammaretroviruses), in which cell division associated with nuclear membrane dissolution is required for infection (33, 42, 62). HIV-1 is also able to infect resting, nondividing T cells in lymphoid tissues (13). These nondividing T cells may contribute to the establishment of protected reservoirs in the host, undermining attempts to eradicate virus-producing cells in the long term (5, 6, 15, 56, 77).

After entry by fusion and uncoating, the viral reverse transcriptase complex traverses the cytoplasm while reverse transcribing the two strands of RNA into DNA (20), forming the viral preintegration complex (PIC). The nuclear envelope forms a barrier that the PIC must negotiate. The nuclear envelope is studded with nuclear pore complexes (NPCs) that form a conduit with a central aqueous channel mediating bidirectional transport of many macromolecules. The NPC corresponds to a 125-MDa structure comprising 50 to 100 polypeptides. Many of these proteins are members of the nucleoporin family characterized by FG repeats (46). During active transport, the central aqueous channel accommodates protein complexes as large as 25 nm in diameter. However, the HIV-1 PIC exhibits a Stokes diameter of 56 nm and represents one of the largest known cargoes successfully transported across the NPC (48). How HIV-1 performs this feat of "molecular gymnastics" remains unknown (66).

All retroviruses contain three major open reading frames, including *gag* (generates the viral core after intravirion processing of the $p55^{gag}$ precursor polypeptide), *pol* (encodes the reverse transcriptase, integrase, and protease enzymes), and *env* (directs the production of the transmembrane and surface glycoproteins). In addition, the primate lentiviruses contain genes for regulatory (*rev* and *tat*) and accessory (*vpr, vpx, vpu, vif,* and *nef*) proteins. Viral protein R (Vpr) is highly conserved in vivo (47, 65) and serves many functions in the viral life cycle yet is frequently lost during in vitro propagation of the virus (25, 80), highlighting an experimental limitation of such in vitro culture systems.

Vpr induces G_2 cell cycle arrest in proliferating human cells when it is overexpressed (1, 25, 28, 60) or in the context of infection with a recombinant vector (37). This effect correlates with the production of herniations in the nuclear envelope (10). Arrest in the G_2 phase of the cell cycle enhances viral replication, in part by increasing the activity of the long terminal repeat (25). Other studies suggest that the prolonged G_2 arrest induced by Vpr promotes apoptosis of the infected cell, perhaps leading to increased virion release and enhanced viral burden (57, 70–72, 78). Although it has been suggested that Vpr inhibits apoptosis early and promotes apoptosis late during the course of HIV-1 infection (9), we have not observed a consistent effect of Vpr on T-cell depletion in infected lymphoid histocultures (14).

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Structural studies performed with full-length, synthetic Vpr indicate that this 96-amino-acid, 14-kDa protein contains a helix-turn-helix domain between residues 17 and 50 and an α-helical stretch between residues 53 and 78 (30). The carboxy-terminal region of Vpr corresponds to an arginine-rich segment that can influence the stability and, potentially, the structure of the entire protein (79). In a previous study, we defined two unique nuclear targeting signals within Vpr: one residing within the arginine-rich carboxy-terminal segment and a second that depends on highly conserved leucines present in the two α-helical regions (35, 64). The distal leucine-rich helix also contains a nuclear export signal (NES) (64). This NES utilizes the chromosome maintenance region 1 protein (CRM1) (17, 52), which binds to the leucine-rich NES directly and mediates export through the NPC in a leptomycin B-sensitive manner (51, 76). However, the biological significance of the nucleocytoplasmic shuttling properties of Vpr remains unknown.

Vpr is predominantly found in the nuclei of HIV-1-infected cells (43), probably reflecting the strength of its two nuclear targeting signals (64). This nucleophilic property of Vpr, coupled with its presence in the viral PIC, led to the observation that Vpr facilitates more efficient HIV-1 replication in nondividing monocyte-derived macrophages (4, 8, 22, 29). In vitro assays further supported a direct role for Vpr in PIC import (58, 59). However, more recent studies have questioned the role of Vpr in the nuclear uptake of the HIV-1 PIC (2) and have also shown that Vpr is not required for HIV infection of nondividing T cells (14). Further, it is unclear how this nucleophilic protein is incorporated into virions, which are assembled in the cytoplasm at or near the plasma membrane.

In this study, we investigated whether the nuclear export function of Vpr contributes to virion incorporation and whether virion Vpr contributes to viral replication in tissue macrophages. For these studies, we have employed HIV molecular clones containing a mutation (L67A) in the distal helix of Vpr that selectively compromises the nuclear export phenotype of Vpr while maintaining its nuclear import and G_2 cell cycle-arresting functions in HIV-1-infected peripheral blood mononuclear cells (PBMCs). We have evaluated the growth of wild-type and mutant viruses in both the T-cell and macrophage compartments of lymphoid histocultures produced with human tonsillar or splenic tissue. HIV infection in the ex vivo lymphoid histoculture system is likely to more closely approximate the conditions encountered in vivo than does infection of mitogen-stimulated PBMCs (23, 24). This tissue-based system is composed of a mixture of HIV-1 targets, including lymphocytes, macrophages, and the supporting cellular network. It requires no addition of cytokines or activating or differentiating agents like those used in more homogeneous primary cellular systems.

MATERIALS AND METHODS

Plasmids. A hemagglutinin (HA) epitope was introduced at the amino terminus of NL4-3 Vpr to form HA-Vpr as previously described (65). For subcellular localization studies, we used a green fluorescence protein (GFP)-pyruvate kinase (PK)-Vpr chimera (64). This GFP-PK-Vpr fusion protein or relevant Vpr mutants allowed subcellular localization by fluorescence microscopy. Since the backbone is larger (≈90 kDa) than the passive diffusion size of the NPC (≈60 kDa), its import and export occur by active transport (52). The nuclear localization sequence (NLS)-GFP-PK-Vpr construct has been characterized (64), and an L67A mutation was derived by cloning the NLS (PKKKRKV) from the simian virus 40 (SV40) large T antigen at the amino terminus of the GFP-PK-VprL67A chimera (64). HIV-1 infection experiments were performed using HIV-1_{107}, composed of an NL4-3 viral backbone modified to contain the V1 to V3 loop from the envelope of the chemokine receptor 5 (CCR5)-dependent primary isolate, Ba-L (73). This modification enables the 107 virus to infect macrophages and $CCR5^+$ $CD4^+$ T cells. The 107 virus containing the VprL67A point mutation was constructed by using paired PCR primers overlapping the *Eco*RI site and a downstream *Nhe*I site located distal to the end of the V3 loop. This amplicon was cloned directly into the digested wild-type viral construct and sequenced to verify that only this single point mutation had been introduced. The construction of the HIV-1 NL4-3 ΔVpr strain has been described previously (14).

Cell cultures, transfections, and microscopic analysis. Expression vector DNA was transfected into HeLa or 293T cells with calcium phosphate. Cells were cultured in Dulbecco modified Eagle medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin G (100 U/ml), and streptomycin (100 μg/ml). All plasmids were transfected with either 4 μg of DNA per well of six-well plates or 3 μg in experiments incorporating 1 μg of an expression vector encoding the red fluorescent protein (RFP) (pDsRed1-N1) (Clontech, Palo Alto, Calif.). Cells plated on coverslips for microscopic analyses were washed with phosphate-buffered saline, fixed for 10 min in 1% paraformaldehyde, and rinsed in water. The coverslips were then inverted and mounted on glass slides using Gel Mount (Biomeda Corp., Foster City, Calif.). Nuclei were visualized with Hoechst 33342 stain (10 μg/ml; Molecular Probes, Eugene, Oreg.) added to the paraformaldehyde. Cells were analyzed with a Nikon TE 300 Quantum fluorescence microscope and a Hamamatsu Orca II charge-coupled device camera.

Heterokaryon analyses. Heterokaryons were generated as described previously (64, 69). Briefly, transfected 293T cells were cultured for 24 h, washed, trypsinized, and replated overnight at a 1:10 ratio with excess untransfected HeLa cells to achieve a total cell concentration of 1.5×10^6 per well. The cells were exposed to cycloheximide (25 μg/ml) for 1 h to prevent de novo protein synthesis and then subjected to membrane fusion by the addition of 50% polyethylene glycol for 3 min. The cells were washed with phosphate-buffered saline (PBS) and incubated for 1 h with cycloheximide. The pDsRed1-N1 vector expressing RFP was also included in the transfections. RFP localizes in the nucleus and cytoplasm of the donor cell and diffuses into the entire cytoplasm of the newly formed heterokaryon, thus delineating its boundaries. However, while RFP is present in the donor nucleus of the heterokaryon, it does not enter the newly fused nuclei (recipient nuclei) during this 2-h procedure. Thus, it is possible to discern readily whether the test protein linked to the GFP shuttles from the donor nucleus (red) to the recipient nuclei (unstained) within the heterokaryon.

Western blot and coimmunoprecipitation analyses. Coimmunoprecipitation experiments were performed by cotransfecting the HA-Vpr vector with Pr55ΔMA-GFP (11), which contains the p6 domain that mediates Vpr binding. Pr55ΔMA-GFP lacks the RNA retention signal in MA that inhibits RNA export and subsequent expression (63). An equivalent number of 293T cells (600,000 cells) were transfected and harvested 48 h later in 500 μl of lysis buffer (50 mM HEPES [pH 7.9], 250 mM NaCl, 0.5% NP-40 detergent, 0.5 mM EDTA supplemented with protease inhibitor [Roche] [1 tablet/ml], 100 μM phenylmethylsulfonyl fluoride). Lysates were aliquoted for direct analysis or incubated with monoclonal mouse antibody HA.11 immobilized on Sepharose Fast Flow beads (Covance) for 15 h at 4°C and then washed three times with lysis buffer. The beads were boiled for 5 min in loading buffer to dissociate any bound proteins before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blotting was performed with monoclonal anti-HA (Boehringer Manheim), polyclonal anti-GFP (Clontech), monoclonal anti-$p24^{gag}$ (NEN) or polyclonal rabbit anti-Vpr (35) antiserum diluted 1:2,000.

PBMC isolation and infection. HIV-seronegative blood was obtained by leukopheresis, and mononuclear cells were isolated on Histopaque-1077, washed with PBS, and activated with phytohemagglutinin (Sigma, St. Louis, Mo.) at 50 μg/ml. After 24 h, the cells were washed and cultured in RPMI supplemented with interleukin-2 (10 U/ml; Roche). Cultures were infected with HIV-1 by resuspending 10^7 cells and 200 ng of viral stocks in 1 ml of medium for 2 h before washing them and culturing them at 10^6 cells/ml.

Cell cycle analyses. Cell cycle experiments were performed with pEGFP expression vector (Clontech) cotransfected into 293T cells at a 1:7 ratio with the indicated HA control or HA-Vpr expression vector. After culture for 24 to 48 h, the cells were trypsinized and fixed for 30 min in 2% formaldehyde. The cells were next washed with PBS and treated with 1 mg of RNase A per ml plus 0.01 mM To-Pro-3 iodide (Molecular Probes) in PBS for 30 min. Cellular DNA content in the transfected (GFP^+) and untransfected (GFP^-) cells was assessed with a FACScan flow cytometer. DNA profiles were analyzed with FlowJo

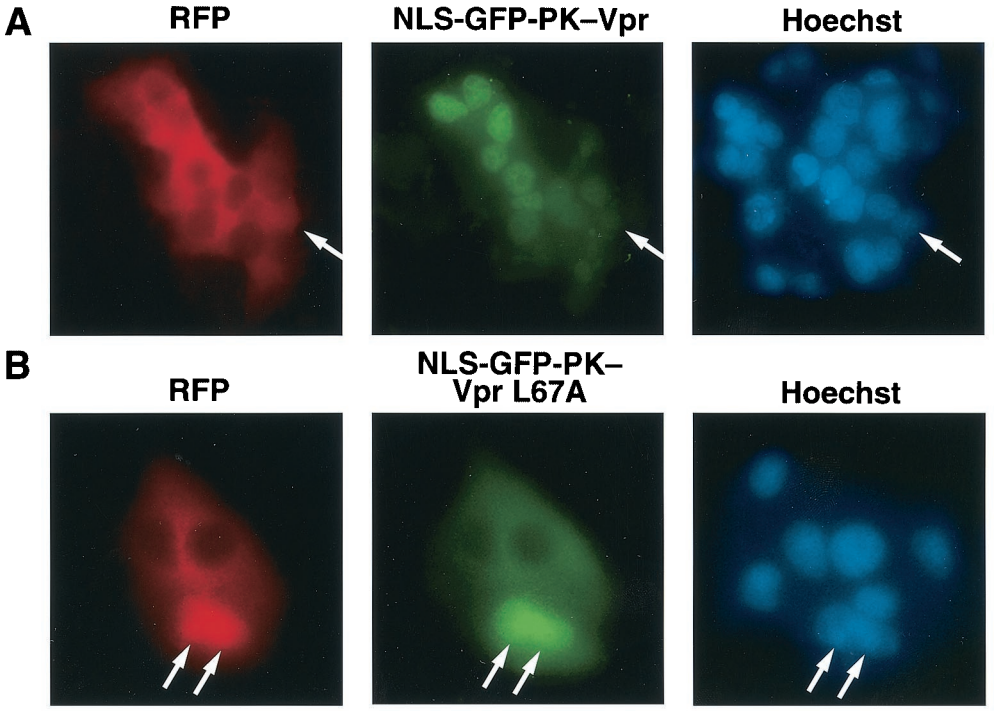

FIG. 1. Identification of a Vpr mutation that segregates nuclear import from nuclear export. The SV40 NLS was cloned into the GFP-PK-Vpr fusion protein to direct this predominantly cytoplasmic shuttling protein into the nucleus. Cells were fused with polyethylene glycol in the presence of cycloheximide (to prevent de novo production of Vpr in the cytoplasm) to examine whether the fusion protein, although on average appearing nuclear, was actually shuttling as indicated by exit from the donor nucleus and accumulation in the recipient nucleus. RFP was included to demarcate the boundaries of the heterokaryon and to mark the transfected (donor) nucleus red (arrows; also shown in the Hoechst staining panel) but not the recipient nuclei (devoid of RFP) introduced by cell fusion. Note that the NLS-GFP-PK-Vpr was able to exit the donor nucleus and accumulate in the recipient nuclei (A) while the L67A mutant was defective for such shuttling (B).

software (Treestar). PBMCs, after 5 days of infection, were processed similarly except that fixation and permeabilization were performed with a solution of 1% paraformaldehyde, 1 mg of human immunoglobulin G per ml, and 0.1% Tween 20 in fluorescence-activated cell sorter buffer (PBS with 2% fetal calf serum). Lymphocytes were analyzed by first gating on live, cycling cells as determined by cell size (forward scatter) and granularity (side scatter) and by DNA content with mock-infected controls. This cycling gate was then interrogated identically between the different infections for intracellular Gag polyprotein expression with the KC57 monoclonal anti-p24 antibody (1:50 dilution) (Coulter).

Preparation of viral stocks. Molecular clones of HIV-1 proviruses were transfected into 293T cells as described above, and the culture supernatants were collected after 48 h. These viral preparations were centrifuged for 10 min at 5,000 rpm in a Beckman GH 3.7 rotor before being aliquoted and frozen. The $p24^{gag}$ concentration was determined by a standard enzyme-linked immunosorbent assay (NEN), and the 50% tissue culture infective dose ($TCID_{50}$) was determined by limiting dilution using pooled, phytohemagglutinin-activated PBMC cultures as targets (23, 55). Virus (300 ng of $p24^{gag}$) was collected by ultracentrifugation at 40,000 × *g* for 90 min to assess virion incorporation of Vpr.

Culture and infection of human lymphoid tissues ex vivo. The ex vivo lymphoid histoculture system was used to more closely approximate HIV infection events occurring in vivo. This system supports viral infection and replication in the absence of exogenous cytokine stimulation and provides the diverse array of cells present in normal lymphoid tissues (23, 26, 55). Noninflammatory spleen or tonsil tissue (obtained from the National Disease Research Interchange) was sectioned into 2- to 3-mm blocks and cultured as described previously (23). Six blocks per well were inoculated with HIV-1 by dropwise addition of 50 $TCID_{50}$. At the indicated times, the medium was collected from the wells to monitor the replication kinetics or the tissue was mechanically disrupted and subjected to flow cytometric analysis as described above for PBMCs. In addition, cells derived from lymphoid tissue were immunostained with various antibodies, including anti-CD3, anti-CD4, anti-CD14, and anti-CD68. Replicates represent data collected from three separate wells.

RESULTS

Segregation of the Vpr NES from the overlapping nuclear import signal. We previously mapped an NES to the leucine-rich domain (LQQLL) of the distal helix spanning amino acids 64 to 68 of Vpr (64). Our mutational analysis further revealed that the L67A analogue was effectively imported into the nucleus but that L64A and L68A Vpr analogues were not. We next investigated the nuclear export properties of the L67A mutant. Wild-type and L67A Vpr were fused at the carboxy terminus of the NLS-GFP-PK chimera, and heterokaryon shuttling studies were performed (Fig. 1). The fusion protein containing wild-type Vpr effectively relocalized into the acceptor nuclei in the heterokaryons, indicating effective shuttling. However, a chimera containing the VprL67A mutant failed to exit the donor nucleus. GFP-PK-VprL67A localizes to the nucleus after addition of the export inhibitor leptomycin B (64) but appears predominantly in the cytoplasm at baseline, indicating that some degree of the nuclear export function is retained (data not shown). However, when measured against the import strength of the SV40 NLS, as shown in these experiments, the nuclear export properties of the L67A Vpr mutant were significantly compromised.

The VprL67A export mutant causes G_2 cell cycle arrest. To assess whether the L67A mutant interferes with the G_2-arresting properties of Vpr, an HA-VprL67A expression vector was

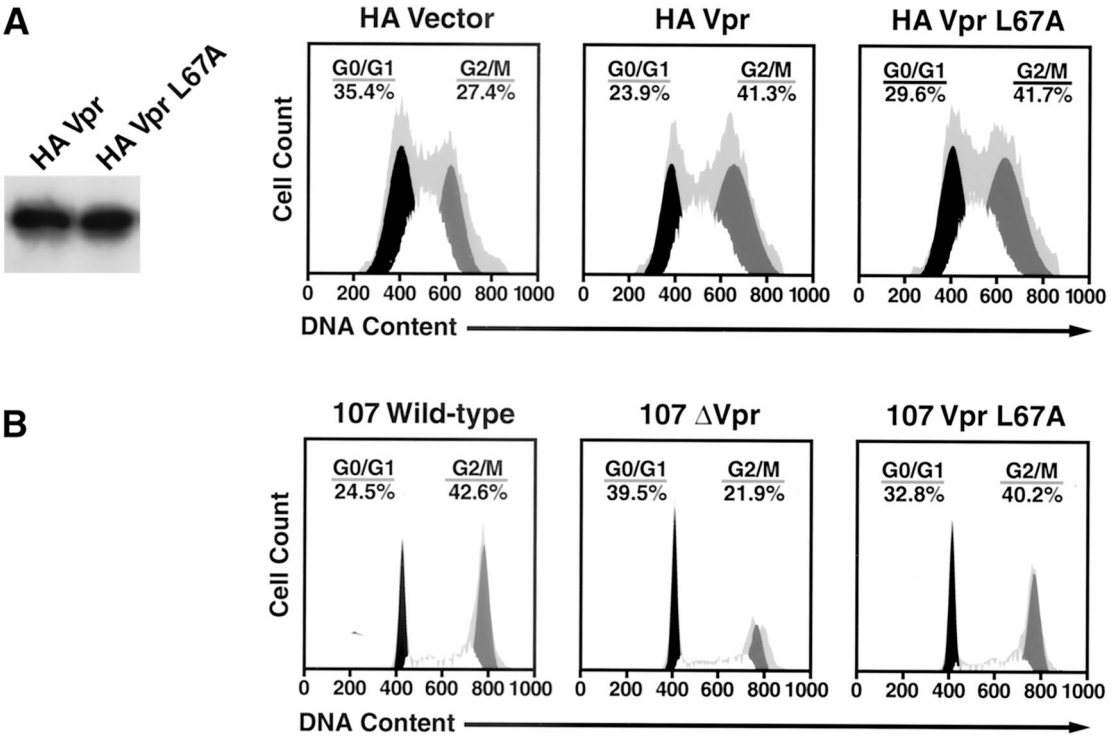

FIG. 2. The VprL67A mutant induces cell cycle arrest. Cells were analyzed for DNA content, and cell cycle profiles were determined with FlowJo software using the Watson Pragmatic model. Primary data are represented by the gray background. (A) Both wild-type Vpr and the L67A-Vpr export mutant were expressed at comparable levels (left panel) in 293T cells and induced a similar accumulation of cells at the G_2/M interface, characterized by a 4*N* complement of DNA. (B) The Vpr L67A export mutant was introduced as a single amino acid change in the HIV-1 CCR5-dependent 107 proviral backbone. PBMCs were infected for 5 days and stained to assess intracellular p24 production, and the infected lymphocytes (anti-$p24^{gag+}$) were analyzed for DNA content. Note the significant accumulation of cells paused or arrested at the G_2/M phase of the cell cycle for lymphocytes infected with the wild-type and VprL67A mutant viruses, compared with the control 107ΔVpr virus.

prepared and introduced into 293T cells. Western blotting revealed that wild-type Vpr and the VprL67A mutant were expressed at comparable levels (Fig. 2A). Cell cycle analysis showed that the levels of G_2 cell cycle arrest were similar in cells transfected with wild-type and L67A export mutant Vpr. To verify that this mutant causes cell cycle arrest at levels achieved during HIV-1 infection of primary cells, we generated proviral molecular clones containing the single VprL67A point mutation or lacking Vpr altogether. Stocks of wild-type 107, 107L67A, and 107ΔVpr generated after transfection of 293T cells were equally infectious as determined by $TCID_{50}$/$p24^{gag}$ content (data not shown). PBMCs were infected with these viruses, and the cell cycle profiles were assessed in the $p24^{gag}$-positive lymphocytes (those productively infected and making the $p55^{gag}$ precursor of $p24^{gag}$) and $p24^{gag}$-negative lymphocytes by analyzing their DNA contents (Fig. 2B). The 107- and 107VprL67A-infected lymphocytes displayed similar increases in the percentage of cells with a 4*N* complement of DNA compared with 107ΔVpr-infected lymphocytes, indicating retention of G_2-arresting properties by the L67A mutant in these primary cells.

Compromise of Vpr export results in decreased incorporation of Vpr into virions. One possible function for Vpr nuclear export is to ensure that there are adequate amounts of this protein in the cytoplasmic compartment to allow its incorporation into new virions. Since 107, 107VprL67A, and 107ΔVpr were equally infectious in PBMC cultures, similar amounts of viral particles, as measured by $p24^{gag}$, were pelleted and examined for virion-associated Vpr (Fig. 3A). Markedly less (<3%) VprL67A than wild-type Vpr was associated with virions. However, these findings do not exclude the possibility that the L67A mutation interfered with the binding of Vpr to the p6 component of the $p55^{gag}$ precursor and, as a result, was not effectively recruited to the virion. Although previous mapping studies indicated that the p6-binding domain of Vpr is located in the helix-turn-helix domain at the amino terminus (44, 45), we performed immunoprecipitation experiments to directly test whether the L67A mutant binds as well as p6 wild-type Vpr does (Fig. 3B). These studies revealed indistinguishable levels of binding of VprL67A and wild-type Vpr to Gag as measured with the Pr55ΔMA-GFP fusion protein. Immunoprecipitation and immunoblotting confirmed that similar amounts of HA-tagged Vpr and Gag proteins were expressed under each of these conditions.

Vpr export is required for efficient infection of macrophages present in human lymphoid tissues. Spleen and tonsil tissue offer an important tool to study the replication of HIV-1 in what appears to be a more physiologically relevant system. As is true for activated PBMC infection, we observed no dependence on Vpr for replication of CXCR4-dependent viruses (14). Consistent with this observation, examination of the number of infected T cells by intracellular anti-$p24^{gag}$ immunostaining showed no dependence on Vpr for CCR5-dependent viral infection of $CD4^+$ T lymphocytes (Fig. 4A). However, when

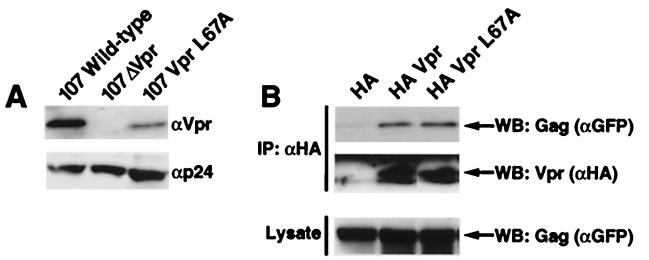

FIG. 3. The Vpr L67A nuclear export mutant is less efficiently incorporated into newly formed virions yet retains the ability to bind to Gag. (A) Virions were collected by centrifugation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Levels of p24 Gag are shown in the lower panel, and levels of intravirion Vpr are shown in the upper panel. Note the substantial decrease (>97%) in the amount of VprL67A associated with the virion compared with wild-type Vpr (top row). (B) HA-tagged Vpr constructs were cotransfected with p55ΔMAGFP. This vector encodes a large segment of Gag, including the p6 domain, but does not require Rev for its expression. Immunoprecipitation (IP, top two rows) of exogenously expressed HA fusion proteins and subsequent immunoblotting (WB) were used to evaluate the interaction of wild-type Vpr and the Vpr L67A analogue with Gag. Expression levels of the Vpr proteins and the Gag fusion protein are shown in the lower two panels. Note the equivalent interaction of VprL67A and wild-type Vpr with the Gag fusion protein (top panel).

the number of HIV-producing macrophages was assessed, a 60% reduction was consistently observed with the CCR5-dependent virus containing the Vpr export mutant (L67A) compared to wild-type virus. Viruses lacking Vpr displayed an equivalent decrease. Since both nuclear import and G_2 cell cycle arrest are intact with VprL67A, these results suggest that nuclear export of Vpr is important for viral spread to macrophages present in lymphoid tissues. Because nuclear export appears to be important for incorporation of Vpr into virions, these data imply that Vpr is required during the initial phase of infection of target macrophages in these tissues. Of note, although macrophages represent only about 1% of the lymphoid tissue, we observed a 67% reduction in the amount of $p24^{gag}$ present in the media of these lymphoid histocultures after infection with virus containing the VprL67A export mutant or lacking Vpr altogether. These findings support the notion that macrophages are an important reservoir that disproportionately contribute to viral burden in these lymphoid tissues (14).

DISCUSSION

This study shows that the nuclear export property of the nucleocytoplasmic shuttling protein Vpr is required for efficient incorporation of Vpr into virions, which is required for efficient HIV-1 replication in tissue macrophages. We studied a Vpr mutant (L67A) that is compromised in its nuclear export property but retains wild-type nuclear import and causes G_2 cell cycle arrest. Using this mutant in the context of a molecular HIV clone to infect lymphoid histocultures derived from human tonsil or spleen tissue, we found that the limited incorporation of the Vpr L67A mutant into virions reduced the number of infected tissue macrophages by 60%. A similar reduction was obtained with viruses lacking the entire Vpr gene. In contrast, the L67A Vpr export mutant had no effect on levels of HIV infection of T cells within the same lymphoid

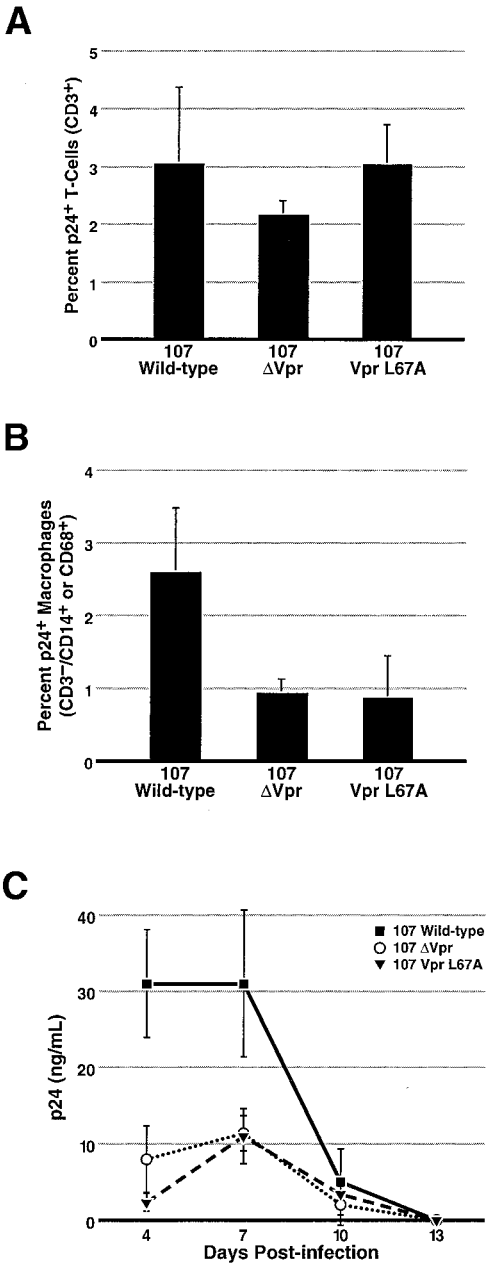

FIG. 4. An HIV-1 molecular clone containing the Vpr L67A export mutant can infect T cells efficiently but is as defective as ΔVpr HIV-1 for infection of tissue macrophages. Lymphoid histocultures prepared from tonsil and spleen (represented here) were infected with 50 $TCID_{50}$ per block of tissue with the indicated CCR5-dependent 107 virus. After 7 to 10 days, the blocks were mechanically dispersed and examined by fluorescence-activated cell sorting using the indicated cell surface markers and intracellular anti $p24^{gag}$ staining. There were six blocks per well, and error bars represent the standard deviation for three wells. (A) There was no difference in the number of infected T cells ($CD3^+$ lymphocytes) in the presence or absence of Vpr. (B) A 60% reduction in the number of infected macrophages ($CD3^-$ $CD14^+$ and/or $CD68^+$) was observed for the ΔVpr and the VprL67A mutants compared with viruses expressing wild-type Vpr. (C) Supernatants of these cultures were colleted over the course of the experiment and analyzed for $p24^{gag}$ levels by enzyme-linked immunosorbent assay. There was a 67% decrease in the amount of secreted $p24^{gag}$ in the cultures infected with the ΔVpr or the VprL67A virus compared with the wild-type virus. These results were reproduced in more than four independent experiments with human spleen and tonsil tissue.

cultures. Nevertheless, we observed a 67% reduction in viral production in these tissues, even though only 1% of the available cellular targets for infection are macrophages. These findings support the notion that both virion-associated Vpr and macrophage targets of viral replication are key factors in the overall HIV-1 load generated in these lymphoid tissues. Both HIV replication in macrophages and PIC import depend upon Vpr. This is further supported by the observation that the HIV-2/simian immunodeficiency virus SM Vpx protein aids in PIC import into the cell nucleus (16) and also enhances infections of macrophages in lymphoid tissues (14). These data also add support to the proposal that infected macrophages significantly contribute to the viral load of HIV-1-infected patients (14, 26, 31, 34, 53).

Vpr is incorporated into the HIV-1 PIC and is thought to facilitate its nuclear import in cooperation with the matrix (MA) (3, 22, 27, 75) and integrase (2, 21) proteins as well as the central DNA flap (81); however, the actual involvement of integrase and the central DNA flap has recently been questioned (12). The specific role of MA in PIC import has also been questioned (19, 50, 61) but may be partially explained by recognition that $p55^{gag}$ is a nucleocytoplasmic shuttling protein (11). Finally, even the role of Vpr during PIC import has been controversial. Vpr facilitates replication in monocyte-derived macrophages, but only when virus is added at a low multiplicity of infection (21). Impaired viral DNA import requires mutations in both Vpr and MA, suggesting at least some redundancy in their functions (29). Others have concluded that Vpr does not contribute to HIV infection of monocyte-derived macrophages at all independently of MA (27) or even with the loss of both these nuclear import signals (40). However, in the more biologically relevant context of lymphoid tissue, Vpr clearly facilitated replication in tissue macrophages (14), and in contrast to other studies (32), its presence in the virion is required for this effect. It is clear that Vpr does not facilitate replication in artificially arrested T cells (2, 8, 27). Further, in our previous work utilizing the CXCR-4-dependent HIV strain NL4-3, we demonstrated that Vpr does not facilitate HIV infection of resting T cells infected in ex vivo lymphoid histocultures (14). Vpr also does not contribute to overall viral production when macrophages are excluded from infection, as seen with NL4-3 infection of lymphoid tissues (14). These findings suggest that Vpr exerts its effects in a cell type-restricted manner.

Jenkins et al. have confirmed the nucleocytoplasmic shuttling properties of Vpr (36). Curiously, in this study, Vpr export was insensitive to leptomycin B even though mutations in the predicted leucine-rich, CRM-1-binding domain abrogate the phenotype (36, 64). Our prior studies demonstrated that leptomycin B impairs nuclear export of Vpr (64). Jenkins et al. also suggested that nuclear export of Vpr was not required for virion incorporation when virions were produced in the presence of cotransfected plasmids encoding a Vpr export mutant. While Vpr is specifically incorporated into the virion through interaction with the p6 portion of the $p55^{gag}$ precursor protein (7, 39, 54, 79), the stoichiometry, once thought to be 1:1 with $p55^{gag}$ in the virion, is now estimated to be as low as 1:7 with capsid (49) or even as low as 14 to 18 Vpr molecules per virion (67). More importantly, the quantity of Vpr in the virion can be greatly enhanced (up to ca. 40-fold) by cotransfecting cells with Vpr and proviral DNA expression plasmids (67, 68). Such overexpression experiments involving Vpr must therefore be interpreted with caution. Specifically, the levels of Vpr achieved in the study by Jenkins et al. (36) may have led to increased cytoplasmic concentrations of Vpr that masked the key function of the NES within Vpr. In our experiments, the VprL67A mutant was cloned into the viral genome and thus was expressed at levels characteristic of wild-type HIV-1 infection. Under these conditions, the Vpr NES is key for full incorporation of Vpr into virions.

The absence of Vpr does not completely prevent the infection of tissue macrophages. Vpr enhances such infections either by acting synergistically with the other import factors or by providing a redundant signal for more efficient import of the PIC across the NPC. It has been suggested that Vpr acts like an importin-β homologue through its direct binding to nucleoporins within the NPC, although this appears to be context dependent (18, 58, 74). While MA and integrase utilize the importin-α/importin-β-dependent pathway of nuclear import, Vpr possesses noncanonical NLSs and is not imported exclusively through these classical mechanisms (21, 22, 35, 38, 64). Thus HIV-1 may have adapted a novel strategy to bypass cellular defense mechanisms targeted at excluding viruses from the nucleus. Importantly, Vpr does not facilitate the infection of resting, nondividing T cells in the same tissue context where macrophage infection is enhanced (13, 14). Therefore, cell-specific factors must dictate whether Vpr is required for or is active in PIC import. In view of the unexpectedly large contribution of infected macrophages to the viral burden in lymphatic tissues, interrupting macrophage-dependent growth by compromising Vpr action in vivo could lead to a sharp decline in the viral burden in infected patients.

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