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Conserved Amino Acids of the Human Immunodeficiency Virus Type 2 Vpx Nuclear Localization Signal Are Critical for Nuclear Targeting of the Viral Preintegration Complex in Non-Dividing Cells

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Abstract
The HIV-2 viral accessory protein Vpx is related to, but distinct from the Vpr protein of HIV-1. Vpx is packaged into virions and as a component of the viral preintegration complex (PIC) is required for efficient virus replication in non-dividing cells. We have previously reported that the minimal transferable region of Vpx that contained karyophilic properties was aa 65 to 72. Analysis of Vpx sequences from various HIV-2/SIV strains reveals that this region contains highly conserved amino acids, including two basic residues (K68, R70) and three tyrosines (Y66, Y69, Y71). Here, we demonstrate that mutation of the basic or tyrosine residues abolishes PIC nuclear import in arrested cells as assessed by PCR detection of viral integration. Examination of cell-free virus by Western blot indicated that all mutant proteins were incorporated into virions, suggesting that the lack of replication in arrested cells was not due to a loss of Vpx in target cells. Together, these studies map critical residues of the Vpx nuclear localization signal that are required for efficient infection of non-dividing cells.

Keywords: HIV-2, Vpx, Preintegration complex, Nuclear localization

Introduction

A hallmark and critical determinant of the pathogenesis of the lentivirus subfamily of retroviruses is the ability to infect non-dividing cells. After reverse transcription, targeting of the viral DNA to the nucleus of cells is mediated by a large nucleoprotein preintegration complex (PIC). This complex, which has been difficult to characterize, is comprised of both cellular and viral components. Likely, only a small number of the cellular proteins associated with the PIC have been identified. Among those identified, only a few, including barrier to auto integration factor (BAF) and HMG Y(I), have been shown to be functionally relevant (Chen and Engelman, 1998, Farnet and Bushman, 1997, Harris and Engelman, 2000, Hindmarsh *et al.*, 1999, Li *et al.*, 2000, and Lin and Engelman, 2003). The viral components include the dsDNA, reverse transcriptase, matrix, nucleocapsid, integrase, and Vpr (HIV-1) or Vpx (HIV-2/SIV) proteins (Bukrinsky *et al.*, 1993, Farnet and Haseltine, 1991, Hansen and Bushman, 1997, Karageorgos *et al.*, 1993, and Miller *et al.*, 1997). HIV-2 contains both the *vpr* and *vpx* genes, each with discrete functions (Fletcher *et al.*, 1996). While Vpx is homologous to HIV-1 Vpr, is packaged into virions, and is a component of the HIV-2 PIC, it lacks a nuclear export signal and any cell-cycle arrest function (Belshan and Ratner, 2003 and Hansen and Bushman, 1997). Therefore, like HIV-1 Vpr, Vpx is necessary for effective nuclear translocation of the PIC, but is dispensable for integration. Furthermore, SIV_{sm} Vpx has been demonstrated to be required for efficient virus dissemination and pathogenesis in vivo (Hirsch *et al.*, 1998).

In addition to its composition, the transport of the PIC remains one of the least well understood aspects of HIV replication. Work by McDonald *et al.* suggests that the PIC associates with and traverses the microtubule network to microtubule organizing centers near the nuclear envelope (McDonald *et al.*, 2002). However, it remains unclear what facilitates PIC transport along the network. Furthermore, once the PIC reaches the nuclear membrane, the sequence of events that result in the nuclear import of the viral ds-

DNA remains unclear. Numerous viral components of the HIV-1 PIC contain one or more karyophilic signals including matrix, integrase, vDNA (central polypurine tract), and Vpr (or Vpx for HIV-2/SIV) (reviewed in Goff, 2001, Sherman and Greene, 2002, and Stevenson, 1996). However, deletion of any single signal results in only a modest reduction of PIC import efficiency. Therefore, these signals do not appear to be synergistic, but rather redundant.

HIV-2 Vpx is a small hydrophobic protein that is a component of the PIC and required for efficient infection of non-dividing cells (Mahalingam *et al.*, 2001, Pancio *et al.*, 2000, and Ueno *et al.*, 2003). The localization of Vpx is context dependent. Vpx has a nuclear localization signal (NLS), but no NES (Belshan and Ratner, 2003). In the presence of unprocessed Gag in a virus producing cell, Vpx is packaged into virions through an interaction with the p6 region of Gag (Jin *et al.*, 2001, Pancio and Ratner, 1998, and Selig *et al.*, 1999). Packaging of Vpx is necessary for its activity during the early stage of virus replication. Expressed in the absence of the Gag polyprotein, Vpx predominantly localizes to the nuclei of cells (Belshan and Ratner, 2003, Mahalingam *et al.*, 2001, and Pancio *et al.*, 2000). We have mapped a minimal transferable nuclear localization signal (NLS) to amino acids 65–72 (Belshan and Ratner, 2003); however, other C-terminal residues have been implicated in regulating Vpx nuclear localization and PIC import in non-dividing cells (Belshan and Ratner, 2003, Pancio *et al.*, 2000, and Rajendra Kumar *et al.*, 2003). In these studies, there exists a strong correlation between the ability of Vpx mutants to localize to the nucleus and the ability of viruses containing those mutations to infect non-dividing cells. Thus, Vpx appears to be a critical regulator of HIV-2/SIV PIC nuclear import.

The goal of our ongoing studies is to characterize the pathway of Vpx nuclear import as a means to dissect the process of PIC transport and nuclear targeting. We have examined the minimal transferable nuclear localization signal of HIV-2 Vpx (…^{65}SYTKYRYL^{72}…). This stretch of amino acids is highly conserved among HIV-2/SIV strains (Figure 1A), further suggestive evidence of the importance of this region. Here, we use mutational analysis to study the Vpx NLS and characterize the role of Vpx in PIC nuclear transport. We define three critical residues (K68, Y69, R70) in the minimal transferable NLS required for HIV-2 infection of non-dividing cells. Mutation of any of these residues did not appear to inhibit Vpx incorporation into virions, suggesting that the NLS is separable from the packaging domain of Vpx. Nor did these mutations impede HIV-2 infection of dividing cells. Thus, we have defined several critical *cis*-acting residues of the Vpx NLS for targeting of the HIV-2 PIC in non-dividing cells.

Results

Mutagenesis of conserved amino acids in the Vpx nuclear localization signal (NLS)

Our previous studies identified two transferable NLSs of Vpx that overlapped at amino acids 65–72 (Belshan and Ratner, 2003). Our next goal was to identify the critical residues of Vpx to further characterize the pathway of Vpx nuclear import and the role of Vpx in preintegration complex (PIC) nuclear targeting. Examination of various HIV-2 and SIV Vpx sequences in the Los Alamos Database indicated that several amino acids within this region are highly conserved among the HIV-2/SIV family (Figure 1A), suggesting they are essential for the function of the protein. In our previous study, we noted that alanine substitution of both basic residues (K68 and R70) and/or the three tyrosine residues (Y66, Y69, Y71), but not the serine residues ablated the nuclear localization of a green fluorescent protein-Vpx fusion protein (Belshan and Ratner, 2003). In the present studies, we wished to determine the effect of these substitutions in Vpx on PIC import. Therefore, we constructed a series of full length HIV-2 ROD10 molecular clones containing these original alanine substitutions in Vpx and also constructed additional Vpx mutants with different combinations of alanine substitutions for the tyrosine residues (Figure 1B).

Infection of MAGI-5 cells with viruses containing alanine substitutions in the Vpx NLS

It has been known for some time that Vpx is dispensable for replication in dividing cells in vitro (Hu *et al.*, 1989, Kappes *et al.*, 1991, Marcon *et al.*, 1991, Shibata *et al.*, 1990, and Yu *et al.*, 1991). To confirm that the molecular clones we

A.

Consensus:	gmSxs**YtKYRYL**cliqkAx
HIV-2 ROD10	---E----------K-V
HIV-2 BEN	---A----------M-K-I
HIV-2 KR	---Q--------R-M-Y-M
HIV-2 EHO	---I----------M---M
SIVsmPBJ1.9	---V----------M
SIVmac239	---P--V----------M
SIVmne	---Q--V----------L
SIVmm9	---E---------Y----L
SIVdrl1FAO	QL-FT-S-----L-L---M
SIVmnd5440	QR-LD-------L-MH--M

B.

	GMSES**YTKYRY**LCIIQKAV
RMX-Δ	
RMX-S-A	--A-A----------
RMX-Y-A	-----A--A-A--------
RMX-KR-A	-------A-A---------
RMX-Y-F	-----F--F-F--------
RMX-66,69A	-----A--A----------
RMX-66,71A	-----A----A--------
RMX-69,71A	--------A-A--------
RMX-66A	-----A----------
RMX-69A	--------A----------
RMX-71A	----------A--------

Figure 1. Conservation of the Vpx NLS and mutants constructed for these studies. (A) Alignment of selected sequences from the Los Alamos HIV sequence database. The consensus sequence is given at the top. Capital letters indicate highly conserved amino acid residues, small-case letters denote dominant amino acid residues, 'x' indicates no dominant residue. (B) Summary of the mutations constructed for the studies presented. RMX-Δ represents a clone in which both methionine codons of *vpx* have been altered to abolish protein translation.

constructed were viable, we assayed the ability of each virus to infect MAGI-5 indicator cells. Viruses were produced by transfection of 293T cells, concentrated, and quantified by p27 ELISA. MAGI-5 cells were infected in 96-well plates with equal amounts of virus (usually 5 ng/well) for 48 h, then fixed and stained for β-galactosidase expression. The virus titer was calculated by the number of blue nuclei. As expected, all viruses, including the *vpx*-deleted virus (RMX-delta) retained the ability to infect dividing cells (Figure 2). With the exception of the triple tyrosine to alanine mutant (RMX-Y-A), all the viruses infected the cells at levels similar to the wild-type ROD10. RMX-Y-A consistently displayed approximately 70% of the level of infection of the wild-type ROD10.

Amino acids 68–70 of Vpx are critical for HIV-2 infection of non-dividing cells

Our previous studies demonstrated that mutation of the basic and tyrosine residues disrupted the ability of GFP-Vpx to localize to the nucleus of cells (Belshan and Ratner, 2003). Next, we wanted to determine the effects of these substitutions on the ability of HIV-2 to infect non-dividing cells. Given the large number of mutants we needed to assay, we developed a short-term arrested-cell assay rather than utilize the traditional Mϕ infection assays. Adapted from the method described by Brussel and Sonigo (2003), this assay detects integrated viral DNA by nested PCR (see Materials and methods). For each experiment, U937 cells were synchronized by serum starvation for 48 h and half of the cells arrested by release into complete medium containing 400 μM l-mimosine. The other half was released into complete medium as a dividing cell control. Growth-arrest of the cells was monitored by propidium iodine staining at the end of the experiment (data not shown). Genomic DNA

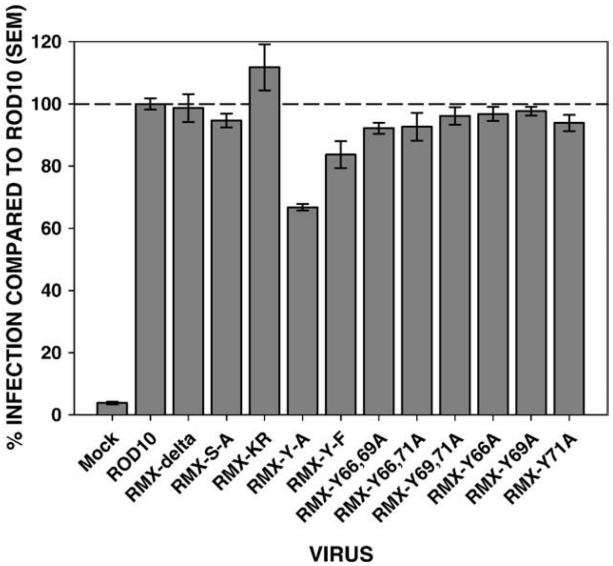

Figure 2. Infection of MAGI-5 cells by viruses containing mutations in Vpx. MAGI-5 cells were infected and virus titer determined as described in Materials and methods. Results are presented as a comparison to the wild-type ROD10 virus and represent at least three independent experiments with at least triplicate infections. Bars denote standard error of the mean.

was isolated at 0, 24, and 48 h post-infection to assess viral DNA integration by nested PCR. The first PCR uses two *Alu* element primers and an HIV-2 LTR-specific primer with a linker. This PCR amplifies LTR-genome fragments of various sizes. The second PCR uses the linker primer with another HIV-2 LTR primer and amplifies a specific product of 242 bp. The second PCR was performed with [^{32}P]TTP for visualization of the product by autoradiography after electrophoresis. Genomic DNA from a known number of CEMx174 cells chronically infected with HIV-2_{ST} (Kong *et al.*, 1988) was used as a positive control in each experiment (data not shown). Each mutant virus was assayed at least three times and the results presented in Figure 3 represent the dominant phenotype observed for each viral mutant.

Each individual experiment contained both ROD10 and the *vpx*-deleted virus, RMX-Δ. As indicated, ROD10 infected the arrested U937 cells only slightly less well than the dividing cells (Figure 3). RMX-Δ repeatedly failed to efficiently infect the arrested U937 cells, but still infected the dividing cells. Therefore, RMX-Δ acted as a negative control in each experiment. Additional controls assayed but not shown included uninfected cells and cells infected with heat-inactivated (65 °C for 30 min) ROD10. Integrated virus was not detected in either of these cells after repeat experiments. Alanine substitution of serine residues 65 and 67 (RMX-S-A) appeared to have no effect on the ability of the virus to infect non-dividing cells. However, alanine substitution of the three tyrosine residues at positions 66, 69, and 71 (RMX-Y-A) or both basic residues (K68, R70-RMX-KR) abolished infection of the growth-arrested cells. Interestingly, these results correlate well with the cell localization phenotypes we previously observed with mutations in GFP-Vpx (Belshan and Ratner, 2003).

The conservation and critical requirement of these tyrosine residues for nuclear localization made it tempting to speculate that phosphorylation of these residues could regulate Vpx function. Although initial studies reported that HIV-2 Vpx was not phosphorylated (Franchini *et al.*, 1988), a recent study demonstrated phosphorylation of SIV Vpx (Rajendra Kumar *et al.*, 2005). However, our own analysis by [^{32}P]phosphoric acid labeling of either virus or over-expressed Vpx failed to detect any phosphorylation (data not shown). Furthermore, to assay whether any such modification to the tyrosine residues was important for PIC import, we constructed a clone in which we substituted phenylalanine at the three tyrosine positions (RMX-Y-F). This virus retained the ability to infect non-dividing cells (Figure 3). These two pieces of evidence suggest that phosphorylation at these tyrosine residues does not regulate HIV-2 Vpx function. Further mutation of the tyrosine residues indicated that Tyr69 alone is critical for the ability of Vpx to mediate infection of non-dividing cells. This is demonstrated by the fact that any clone containing the Y69A substitution (RMX-66,69A, RMX-69,71A, RMX-69A) was unable to efficiently infect growth-arrested cells. Together, the results from this series of experiments indicate that amino acids 68–70 of HIV-2 Vpx are critical for the infection of non-dividing cells.

We also determined the cell localization phenotype of GFP-Vpx fusion proteins containing the tyrosine to ala-

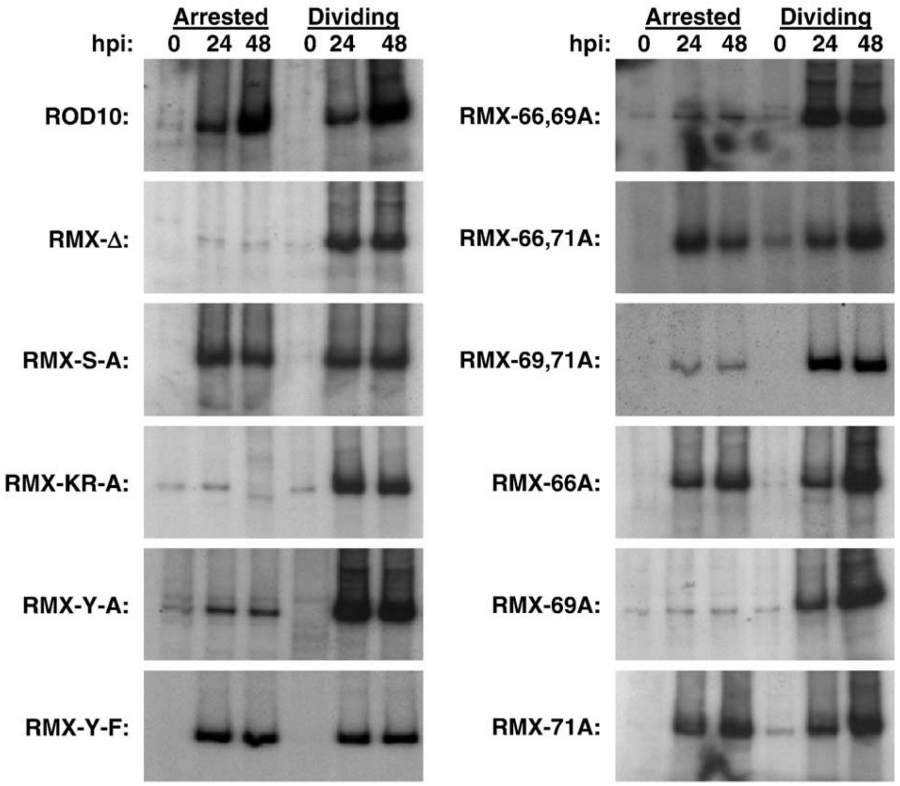

Figure 3. Ability of *vpx*-NLS mutant viruses to infect arrested and dividing U937 cells. U937 cells were growth arrested and infected as described in Materials and methods. Cells were infected with viruses containing the indicated mutations in *vpx*. At the times noted, genomic DNA was harvested and the integration of viral DNA detected by the nested PCR method described in the Materials and methods. Each mutant was assayed at least three times and the panel presented for each mutant represents the dominant phenotype. Control PCR assays for the detection of β-globin indicated similar levels of genomic DNA (±10%) were isolated among the samples (data not shown).

nine substitutions. Similar to the pattern of results we previously observed, the fusion proteins containing the three tyrosine to alanine substitutions (GFP-X-Y-A) or the Y69A mutation alone (GFP-X-Y69A) displayed a loss of nuclear accumulation compared to the wild-type GFP-Vpx fusion protein (GFP-X) (Figure 4). The other constructs containing the Y69A substitution (Y66,69A and Y69,71A) also lost nuclear accumulation (data not shown). Collectively, these and our previous localization results (Belshan and Ratner, 2003) demonstrate that amino acids 68–70 are critical for nuclear localization of Vpx in addition to the ability of HIV-2 to infect non-dividing cells.

The inability of Vpx NLS mutants to infect non-dividing cells does not result from a loss of virion associated protein

Vpx is dispensable for replication in dividing, but not non-dividing cells. To confirm that the defective phenotypes observed in the non-dividing cell infection assay did not result from an absence of Vpx in the virions and subse-

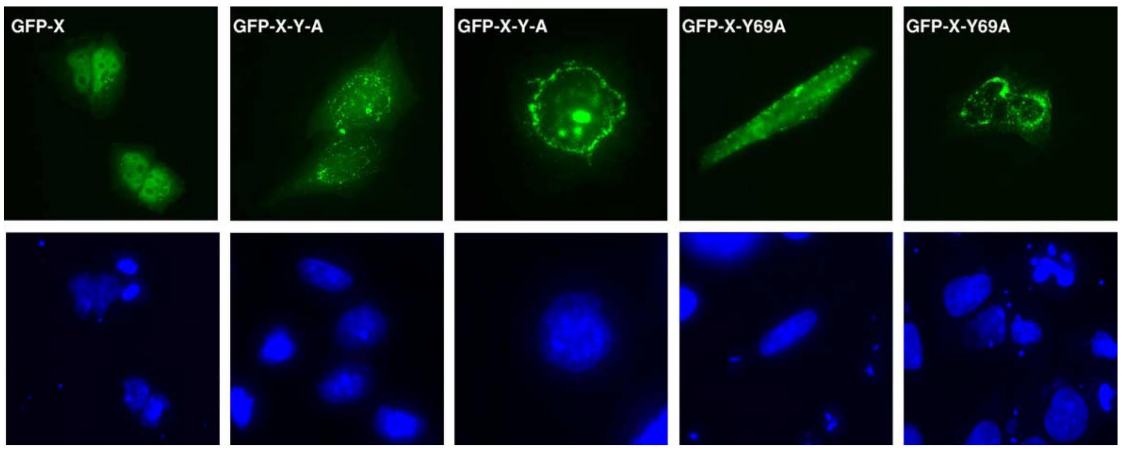

Figure 4. Alanine substitution of Tyrosine 69 in the Vpx NLS abolishes nuclear accumulation of GFP-Vpx. HeLa cells were transiently transfected with the indicated GFP-Vpx fusion protein constructs. 24 h later, the cells were fixed, permeabilized, stained with DAPI (lower panels), and imaged with a fluorescence microscope.

quent PICs, we examined whether the mutant viruses observed to be incompetent to infect non-dividing cells incorporated the mutant Vpx proteins into virions. Virus was produced by transient transfection of 293T cells. 48 h post-transfection, both the supernatants and cell lysates were harvested. Virus in the supernatants was concentrated by ultracentrifugation through a 20% sucrose cushion (w/v in PBS). Vpx and p27 were detected on separate blots using monoclonal antibodies (Figure 5). Vpx was detected in all the mutants except for the vpx-deleted mutant, RMX-Δ, indicating that none of the substitutions were deleterious for Vpx incorporation into virions. The results were not surprising as previous studies indicate that the determinants of HIV-2 Vpx packaging map to amino acids 74–86 (Jin *et al.*, 2001). These results indicate that the failure of the viruses containing the basic or tyrosine mutations to infect non-dividing cells is not due to an absence of Vpx in the incoming virus particles.

Discussion

HIV-2 Vpx is thought to have arisen as a gene duplication of Vpr, and similar to HIV-1 Vpr Vpx is required for efficient PIC nuclear import of non-dividing cells (Fletcher *et al.*, 1996, Sharp *et al.*, 1996, Tristem *et al.*, 1990, and Tristem *et al.*, 1998). Indeed, Vpx and Vpr sequences comprise a Protein Family (Pfam) domain (PF00522) in their central regions (aa 5–99) (Bateman *et al.*, 2004), suggesting likely structural and functional conservation among these proteins. Therefore, it has been proposed that Vpx uses a method of nuclear import similar to Vpr. Since Vpr has been shown to interact with importin α (Kamata *et al.*, 2005, Popov *et al.*, 1998b, and Vodicka *et al.*, 1998) and other

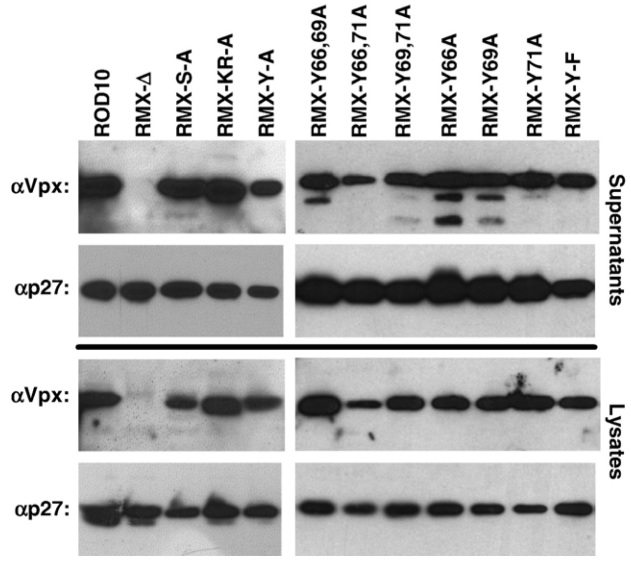

Figure 5. Incorporation of mutant Vpx proteins into virions. Virus was produced by transient transfection of 293T cells and virus collected for 48 h. After the final collection, the cell lysates were harvested, then the supernatants precleared and the virus concentrated by ultracentrifugation through a 20% sucrose cushion. Proteins were separated by SDS-PAGE and Vpx and the p27 capsid proteins detected using specific antibodies as indicated.

nucleoporins (Popov *et al.*, 1998a and Vodicka *et al.*, 1998), it is likely that Vpr enters the nucleus via the nuclear pore. Because of their similarities, we initially hypothesized that Vpx likely utilizes a similar method of nuclear entry as Vpr. While still a viable hypothesis, several findings suggest that Vpr may not be a model system for Vpx. We have identified amino acids 65–72 as a minimal NLS of HIV-2 Vpx, and a larger domain was confirmed to contain an NLS by Kumar *et al.* (Belshan and Ratner, 2003 and Rajendra Kumar *et al.*, 2003). Further confirmation of the role of this Vpx domain in nuclear import would involve studies of the effect of mutations described here on HIV-2 replication in macrophages. However, the parental virus used in this study, ROD-10, replicates inefficiently in macrophages (data not shown). Thus, transfer of these mutations to a different HIV-2/SIV proviral clone will be required for these future studies.

There are two points of interest concerning this region of Vpx. First, this region is not homologous to the Vpr NLS and lacks any recognizable classical NLS. Second, although this region is highly conserved among numerous HIV-2/SIV isolates and Vpx and Vpr constitute a Pfam, a predicted structural alignment of Vpx with HIV-1 Vpr the region of Vpx containing the NLS (aa 65–72) shows weak conservation relative to the highly conserved core domain spanning aa 23–87. The smaller region is predicted to loop out relative to the three helix structure in Vpr (Figure 6). Together, this demonstrates the significance of the NLS region of Vpx, but suggests that the function(s) associated with this region is distinct from Vpr. Furthermore, several homology queries of this region with internet databases yielded no matches. These data support the possibility that Vpx utilizes a unique method of nuclear import, dissimilar to that of Vpr. Indeed, we have performed several yeast two-hybrid screens with Vpx in attempt to identify the pathway Vpx interacts with to access the nucleus. In addition, we also assayed Vpx with a battery of nuclear pore proteins and importins using the yeast system. While our attempts have yielded no positive interactions (data not shown), they highlight the differences between HIV-1 Vpr and Vpx and suggest a novel method of nuclear transport for Vpx.

Numerous mutations in the Vpx C-terminus have been identified that disrupt infection of non-dividing cells. A loss of Vpx function in PIC nuclear targeting may result from several factors, including, but not limited to either a block of Vpx incorporation into virions, a disruption of PIC nuclear import, abolishment of Vpx association with the PIC, or a loss of interaction with an unknown cellular factor required for PIC import. Interestingly, to date, all of the mutations in Vpx that disrupt PIC nuclear import or infection of non-dividing cells have been shown to be nuclear localization defective. This strong correlation suggests that the critical function of Vpx in PIC nuclear transport is nuclear localization. However, there still remains the question of the importance of other residues in the C-terminus of Vpx. Amino acids identified as critical for Vpx nuclear import and HIV-2/SIV infection of non-dividing cells include residues within and without the characterized NLS, such as aa 74–80, H82, G86, C87, and the proline-rich region (101–109) (Belshan and Ratner, 2003, Mahalingam *et al.*, 2001, and Pancio *et al.*, 2000). Any of these mutations may del-

eteriously affect Vpx structure, Vpx association with the PIC, or the interaction of Vpx with a yet-to-be identified cellular factor. Indeed, none of the Vpx mutants that disrupt PIC nuclear import have been examined for their ability to associate with the PIC. Further structure/function studies must be undertaken to delineate the role of these residues in PIC nuclear transport. We observed one result that directly contrasts another report indicating that triple tyrosine to alanine mutation in SIV Vpx ablates virion incorporation but has no effect on nuclear localization of Vpx (Mahalingam *et al.*, 2001). It is presently unclear to us why we observe a contradictory phenotype, but this may be the result of disparate methods or differences between HIV-2 and SIV packaging of Vpx. Further studies are required to delineate the observed differences.

The Vpr/Vpx proteins are considered an important component of the karyophilic signal of the HIV preintegration complex (PIC). The current data suggest that loss of HIV-2/SIV Vpx is more deleterious than the loss of HIV-1 Vpr (Connor *et al.*, 1995, Haffar *et al.*, 2000, Heinzinger *et al.*, 1994, Mahalingam *et al.*, 2001, Pancio *et al.*, 2000, Ueno *et al.*, 2003, and Yu *et al.*, 1991); however, additional and more sensitive assays with HIV-2/SIV need to be performed to bolster this conclusion. Similarly, it has been demonstrated in vivo that deletion of Vpx reduces virus dissemination and productive infection in pigtailed macaques (Hirsch *et al.*, 1998). Combination of the in vitro and in vivo data supports the idea that Vpx is a critical karyophilic component of the HIV-2/SIV PIC. However, it should be noted that neither integrase and MA NLS mutants, nor cPPT mutants have yet to be assessed for SIV in vivo. Such studies would likely clarify the in vivo contribution of each putative karyophilic signal and further our understanding of PIC nuclear targeting.

Materials and methods

Molecular clone construction and virus production and analysis

ROD10 was a kind gift of Klaus Strebel (Bour *et al.*, 1996). Mutagenesis of *vpx* was performed using a shuttling vector containing a modified *Sac*I–*Sac*I fragment (nt 5284–5876) of ROD10 in pGEM-T (Promega, Madison, WI). The modification removed the naturally occurring *Sac*I restriction site at nt 5700. This was accomplished by PCR overlap extension mutagenesis and did not alter the *vpr* ORF. The final plasmid (pX-CC) was used to construct the series of mutations in *vpx* by overlap extension PCR. Mutagenesis was confirmed by sequencing and then the fragment was digested and ligated into the parental pROD10. The final clones were also confirmed by sequencing. The sequences of all primers were designed from the Genbank sequence of ROD10 (accession # X05291). The sequence of these oligos and those used for the mutagenesis are available upon request.

Virus stocks were produced by transient transfection of 293T cells. 1×10^6 293T cells were seeded in 10 cm^2 plates. 24 h later, the media were changed and the cells were transfected with 20 μg of the plasmid molecular clone using TransIT-LT1 as described by the manufacturer (Mirus Bio, Madison, WI). Every 24 h the media were harvested and replaced with fresh media. After 72 h, the virus-containing media were clarified and then concentrated by ultracentrifugation (26,000 rpm for 90 min in a Beckman SW28.1 rotor) through a 20% sucrose cushion. After centrifugation, the media and sucrose were removed and the pelleted virus resuspended in OptiMEM (Invitrogen, Carlsbad, CA), aliquoted, and stored at −80 °C. Virus was quantified by p27 ELISA as described by the manufacturer (Beckman-Coulter, Miami, FL).

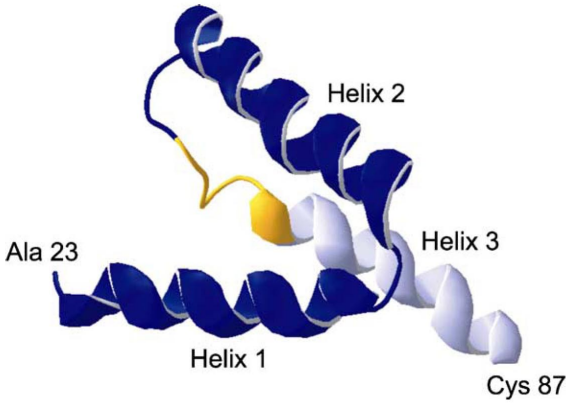

Figure 6. Prediction of helical structure in Vpx. Amino acids 23–87 of Vpx were modeled against the HIV-1 Vpr structure (Morellet *et al.*, 2003) using Robetta/Rosetta (Kim *et al.*, 2004, Chivian *et al.*, 2003, and Rohl *et al.*, 2004). Helix 1–3 indicates predicted a-helices at aa 24–37, 42–59, and 72–86, respectively. The predicted region containing the NLS, aa 60–71, is in orange between helix 2 and 3. The figure was rendered with DeepView (Guex and Peitsch, 1997).

Detection of Vpx in virions

Viruses were produced in the manner described above for analysis of Vpx incorporation. However, after the 48 h harvest, the virus was clarified and pelleted through 20% sucrose. At this time, the cells were also harvested, washed, and lysed with 0.5 mL M-PER solution (Pierce Biotechnology, Rockford, IL). Protein concentrations of the cell lysates were determined by BCA protein assay as described by the manufacturer (Pierce Biotechnology). The pelleted virus was resuspended directly into 0.25 mL 1× SDS-PAGE sample buffer. Vpx and p27 capsid were detected by SDS-PAGE and Western blotting as follows: Either 20 μL of pelleted virus or 20 μg of cellular protein per lane was separated by SDS-PAGE, then blotted to PVDF. Western blots for Vpx were performed using a monoclonal anti-Vpx antibody (Kappes *et al.*, 1993) followed by an HRP-conjugated anti-mouse secondary antibody (Amersham Biosciences, Piscataway, NJ) and was detected using the ECL Plus system as described by the manufacturer (Amersham Biosciences). The p27 capsid protein was detected using the AG3.0 anti-p24 monoclonal antibody (Simm *et al.*, 1995) and an HRP-conjugated anti-mouse secondary antibody.

GFP fusion protein localization

GFP-Vpx is described elsewhere (Pancio *et al.*, 2000). Mutations were introduced into GFP-Vpx by PCR overlap extension and confirmed by sequencing. HeLa cells were maintained and transfected similar to 293T cells as described above. The transfection, fixation, DAPI stain-

ing, and imaging of cells were performed as previously described (Belshan and Ratner, 2003). Images presented represent the dominant phenotype observed.

Virus infections

The MAGI-5 indicator cell line has been described elsewhere (Pirounaki *et al*., 2000). The cells were maintained in DMEM supplemented with 10% fetal calf serum, 4 mM l-glutamine, 1 mM Na-pyruvate, and 100 μg/mL penicillin/streptomycin. To assay virus infection, MAGI-5 cells were seeded at 0.4×10^4 cells/well in a 96-well plate. The next day, the cells were infected in triplicate with 1, 5, and 25 ng of virus in the presence of 20 μg/mL of DEAE-dextran. Forty-eight hours later, the cells were fixed and stained for the expression of β-galactosidase. The number of blue nuclei was counted to determine the level of infection. Results are presented as a percentage of the level of infection of the parental ROD10 and represent a total of at least three independent experiments (therefore 9+ wells for each virus).

U937 cells were maintained in RPMI supplemented with 15% fetal calf serum, 4 mM l-glutamine, and 100 μg/mL penicillin/streptomycin. For the experiments examining the infection of arrested cells, 48 h prior to infection, the cells were pelleted, washed, and resuspended in serum-free RPMI (still containing l-glutamine and antibiotics) to arrest and synchronize the cells. Immediately prior to infection, the cells were counted and resuspended at 1×10^6 cells/mL in either complete RPMI (dividing) or complete RPMI containing 4 μM l-mimosine to arrest cells. 4 mL of cells (4×10^6) was aliquoted into one well of a 6 well plate for each virus, plus one additional well of dividing and non-dividing cells for cell-cycle analysis after the infections were completed. Cells were then infected with 20 μg of virus that had been treated with Turbo DNaseI (Ambion, Austin, TX) for 30 min at 37 °C. At 0, 24, and 48 h post-infection, 1 mL of cells ($\sim 1 \times 10^6$ cells) was removed. Cells were pelleted, the media removed, and the cells washed, and resuspended in 0.2 mL PBS. RNA-free, genomic DNA was isolated using the DNeasy method as described by the manufacturer, Qiagen (Valencia, CA). After the final time point, cell-cycle analysis was performed to confirm that the cells treated with l-mimosine were arrested using the CycleTEST PLUS DNA Reagent kit as described by the manufacturer (Becton Dickinson, San Jose, CA).

Nested Alu PCR

Detection of integrated proviral DNA was performed using a nested PCR method adapted from Brussel and Sonigo (2003). The initial PCR used 500 nM of HIV-2 LTR specific primer with a linker (underlined) and two *Alu* repeat element primers to amplify a pool of products from the genomic DNA. The HIV-2 primer (LROD13) sequence was 5′-ATGCCACGTAAGCGAAACTGCGAGGCTGGCAGATTGAGCCCTG-3′. The first *Alu* primer was 5′-TCCCAGCTACTGGGGAGGCTGAGG-3′; the second was 5′-GCCTCCCAAAGTGCTGGGATTACAG-3′. A 20 μL volume PCR reaction was performed using a 1× JumpStart REDTaq reaction (Sigma, St. Louis, MO) with 4 μL of the genomic DNA sample as a template. After an initial 8 min 95 °C incubation to activate the enzyme, 12 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min were performed. The second PCR was performed with 2 μL of the initial PCR using a 1× JumpStart REDTaq reaction supplemented with 0.1 μCi [32P]α-TTP per 20 μL reaction. For this reaction, 500 nM of the linker primer (5′-ATGCCACGTAAGCGAAACTGC-3′) and an HIV-2 U5-specific primer (5′-TTACTCAGGTGAACACCGAATGACCAGGC-3′) were used. After an initial 8 min 95 °C incubation to activate the enzyme, the reaction conditions for the second PCR were 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. This PCR resulted in the amplification of a 242 bp product. To visualize the products, 10 μL of each reaction was electrophoresed through an 8% native TBE-polyacrylamide gel. After electrophoresis, the gel was dried and exposed to autoradiography film. Unfortunately, the nested PCR method combined with the [32P]TTP labeling resulted in an occasional background band. It was for this reason we analyzed for integration at both 24 and 48 hpi. Each mutant was also assayed independently at least three times. The results present the dominant phenotype observed.

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