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Identification of the Nuclear Localization Signal of Human Immunodeficiency Virus Type 2 Vpx

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Abstract

The Vpx protein of human immunodeficiency virus type 2 (HIV-2) is a viral accessory protein 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), Vpx is required for efficient virus replication in nondividing cells. Therefore, the localization of Vpx in cells is dynamic and dependent upon discrete domains of the protein. Expressed in the absence of other viral proteins, Vpx localizes to the nucleus of cells. However, if expressed with the Gag protein of HIV-2, Vpx localizes to the plasma membrane of cells. To further understand the regulation of Vpx localization, we fused regions of Vpx to β-galactosidase to identify regions of the protein sufficient to mediate nuclear localization. The minimal transferable region of Vpx that conferred nuclear localization in these assays was aa 65 to 72. Alanine substitution of K^{68} and R^{70} in a GFP-Vpx construct abolished nuclear localization, suggesting that the basic residues in this region are important for nuclear import. Analysis of the membrane transport of several GFP-Vpx alanine mutants demonstrated that while separable, the domains of Vpx required for nuclear localization are not distinct from the domains required for membrane transport. The results of heterokaryon shuttling assays indicated that Vpx is not a shuttling protein; however, HIV-2 Vpr did shuttle similar to HIV-1 Vpr.

Introduction

Productive infection of nondividing cells by the human and simian immunodeficiency viruses (HIV and SIV, respectively) requires active nuclear transport of the viral DNA across the host cell nuclear membrane prior to viral dsDNA integration into the host genome. This task is accomplished through the viral preintegration complex (PIC), a nucleoprotein complex composed of cellular and viral components (reviewed in Fouchier and Malim 1999). The viral components include the dsDNA, reverse transcriptase, matrix, nucleocapsid, integrase, and Vpr (HIV-1) or Vpx (HIV-2/SIV) proteins (Bukrinsky *et al.* 1993b, Farnet and Haseltine 1991, Hansen and Bushman 1997, Heinzinger *et al.* 1994, and Miller *et al.* 1997). The matrix, integrase, Vpr, and Vpx proteins have each been identified as having karyophilic properties (Bukrinsky *et al.* 1993a, Gallay *et al.* 1997, Heinzinger *et al.* 1994, and Pancio *et al.* 2000). Although each of these proteins has been demonstrated to be a crucial regulator of nuclear import of the PIC, it remains unclear as to the exact contribution each protein plays. In addition, the central DNA flap has also been shown to be an important signal for nuclear entry of the PIC (Zennou *et al.* 2000).

There is significant sequence homology among the Vpr and Vpx proteins (Tristem *et al.* 1990 and Tristem *et al.* 1998). Both are of similar size (14–17 kDa), are packaged in virions, and possess similar functions (Fletcher *et al.* 1996). The Vpr protein of HIV-1 has been extensively studied and demonstrated to possess at least two discrete functions. Early in virus replication, Vpr mediates infection of nondividing cells by directing nuclear import of viral PICs (Popov *et al.* 1998). Vpr contains two discrete NLSs that directly target to the nuclear pore complex (NPC) and facilitate entry into the nucleus (Jenkins *et al.* 1998). It has also been proposed that Vpr-induced herniation of the nuclear envelope structure may facilitate PIC import (De Noronha *et al.* 2001). Vpr has also been demonstrated to cause G2 cell-cycle arrest of proliferating cells (Goh *et al.* 1994). This effect may contribute to increased

virus production since viral transcription is most active during this phase of the cell cycle (Goh *et al.* 1994). Interestingly, two nonarresting mutants of Vpr failed to alter nuclear membrane morphology, suggesting that nuclear membrane disruption may contribute to G2 cell-cycle arrest (De Noronha *et al.* 2001). HIV-1 Vpr has also been demonstrated to be a shuttling protein capable of nuclear import and export (Sherman *et al.* 2001). While the role of shuttling on Vpr function has not been elucidated, it has been shown to be dispensible for packaging of Vpr into virions (Jenkins *et al.* 2001). Mutation of the nuclear export signal of Vpr abolished Vpr-mediated nuclear membrane herniation (De Noronha *et al.* 2001). Together, these results suggest that Vpr shuttling may be important for Vpr-mediated cell-cycle arrest.

Unlike HIV-1, HIV-2 contains both the *vpr* and the *vpx* genes, each with discrete functions (Fletcher *et al.* 1996). HIV-2 Vpr has been demonstrated to arrest cells in G2, but plays no role in nuclear import of the PIC (Fletcher *et al.* 1996). Vpx is a 17-kDa, 112 amino acid (aa) protein that is packaged into virions but is not crucial for virion morphology. HIV-2 Vpx is necessary for efficient replication in nondividing cells (Pancio *et al.* 2000). As a component of the HIV-2 PIC, Vpx, similar to HIV-1 Vpr, has been shown to be necessary and sufficient for nuclear translocation of the PIC, but plays no role in integration (Hansen and Bushman 1997). As such, Vpx plays an important role in the early steps of virus replication. Indeed, SIV_{sm} Vpx has been demonstrated to be required for virus dissemination and pathogenesis in vivo (Hirsch *et al.* 1998). This may be due to the nuclear import role of Vpx in allowing infection of quiescent cells. The cellular localization of Vpx is dynamic and dependent upon discrete domains within the protein. Expressed in the absence of other HIV-2 components, Vpx exhibits a predominantly nuclear localization, with limited perinuclear localization (Mahalingam *et al.* 2001 and Pancio *et al.* 2000). However, expressed in the presence of HIV-2 Gag, Vpx is observed to localize to the plasma membrane (Pancio *et al.* 2000). The interaction of Vpx and Gag facilitates the packaging of Vpx during virus assembly. Plasma membrane localization and packaging of Vpx are mediated through the interaction of aa 81–86 of Vpx with aa 15–40 of the HIV-2 p6 protein (Jin *et al.* 2001 and Selig *et al.* 1999).

Previous work suggested that the C-terminus of Vpx contained an NLS (Pancio *et al.* 2000). These studies demonstrated that deletion of the 11 C-terminal aa of Vpx abolished nuclear localization of a GFP-Vpx fusion, resulted in decreased nuclear import of viral PICs, and decreased virus replication in macrophage cultures. These studies were confirmed in another article (Mahalingam *et al.* 2001). Using GFP fusion proteins, this study also identified an NLS in a broad region of Vpx (aa 60–85) (Mahalingam *et al.* 2001). Several mutations in this region, including both charged (H82) and hydrophobic residues (L74 and L75), abolished nuclear localization. Other C-terminal mutations were also found to be deleterious to Vpx nuclear localization, further suggesting the C-terminus of Vpx (aa 60–112) is required for full nuclear targeting capability. Here we report that aa 65 to 72 is the minimal transferable region of Vpx that contains nuclear localization properties. Furthermore mutation of basic residues in this region disrupted nuclear localization of a GFP–Vpx fusion, but was not deleterious to membrane transport in the presence of Gag- Pol. These results demonstrate that the nuclear localization and membrane transport of Vpx are separable. However, other mutations C-terminal to this region in Vpx disrupted both nuclear localization and membrane transport, indicating that the requirements for both functions are not discrete. Defining the mechanism of Vpx nuclear import may lead to the development of therapeutic interventions at early stages of virus replication.

Results

Construction and expression of lacZ–vpx fusions

Published reports conflict as to the exact size limit (40–60 kDa) of proteins capable of passive diffusion between the nucleus and cytoplasm of cells. Therefore, to identify the domain(s) of Vpx that facilitates nuclear localization, we constructed a series of *lacZ–vpx* fusions. The use of β-galactosidase (β-gal) as a fusion partner for the Vpx fragments resulted in proteins greater than 100 kDa in size, which

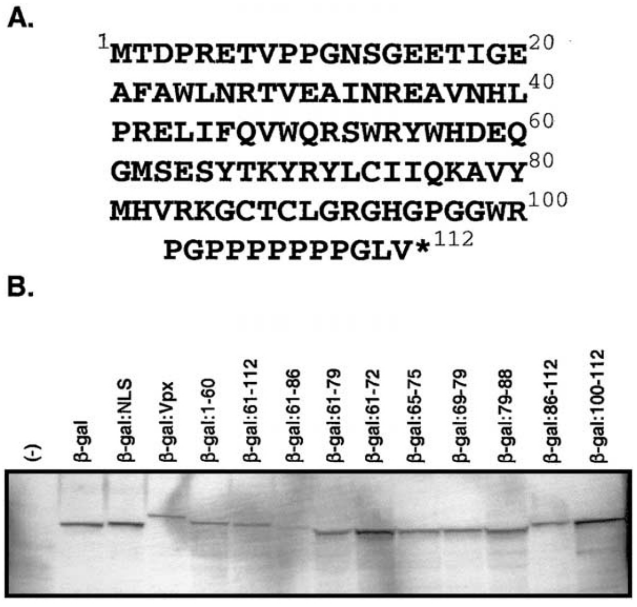

Figure 1. Amino acid sequence of HIV-2 ROD Vpx and expression of β-gal–Vpx fusion proteins. (A) Amino acid sequence of HIV-2 ROD Vpx. (B) Western blot of lysates from 293T cells transiently transfected with indicated *lacZ–vpx* fusions (numbers indicate aa of Vpx present in fusion). Fusion proteins were detected using a monoclonal anti-β-gal antibody followed by an alkaline phosphatase conjugated anti-mouse secondary antibody. Antibody binding was detected using Western blue (Promega) colorimetric substrate.

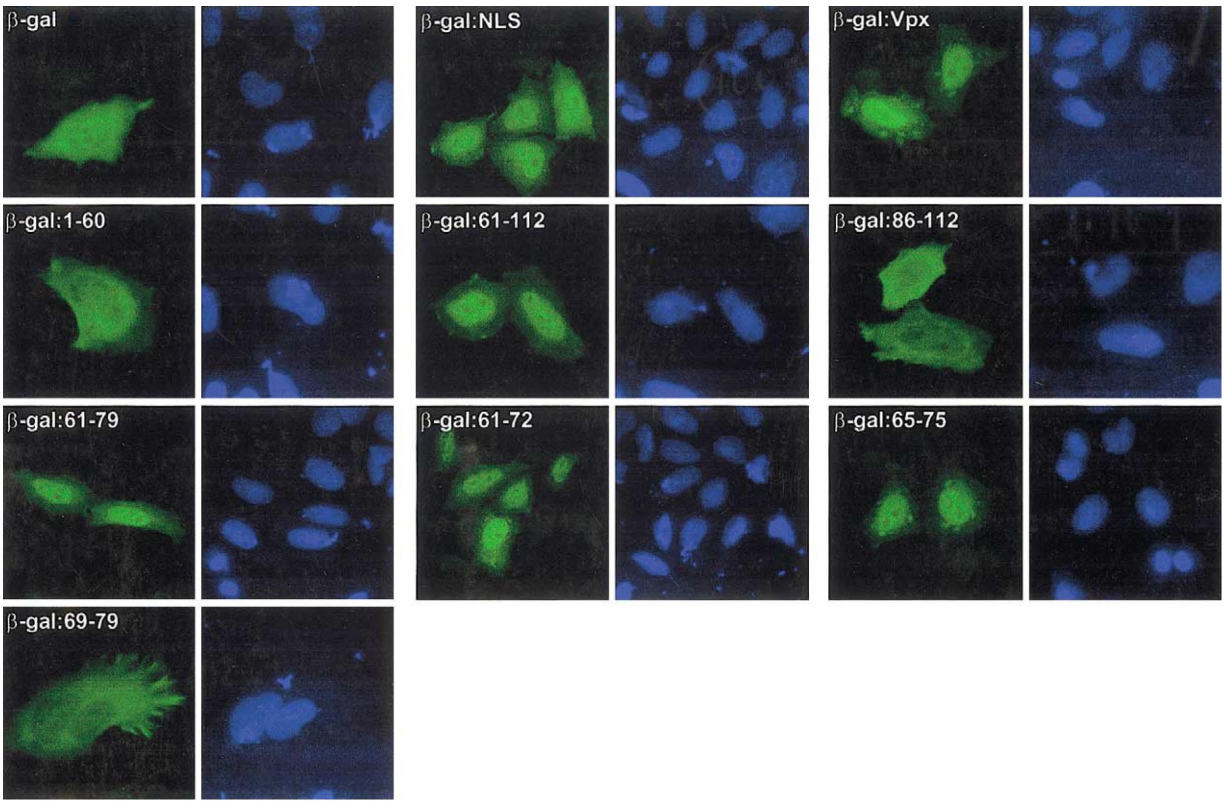

Figure 2. Subcellular localization of β-gal–Vpx fusion proteins. β-gal–Vpx fusion proteins were detected in transiently transfected HeLa cells by indirect immunofluorescence as described under Materials and methods. The regions of Vpx contained in each fusion are given. Nuclei were visualized by staining with DAPI and are shown in panels to the right of the immunofluorescence images.

would require active transport to move into and out of the nucleus. Full-length and smaller regions of *vpx* (Figure 1A) were inserted at the 3′ end of the *lac*Z gene in a eukaryotic expression vector pCR3.1 (Invitrogen). The nuclear localization signal (NLS) of the large T antigen of SV40 was used as a positive control. Expression of these β-gal–Vpx fusion proteins was detected by Western blot of transiently transfected 293T cells using an anti-β-gal monoclonal antibody (Promega) (Figure 1B). All proteins were of the predicted size.

Amino acids 61 to 75 of Vpx facilitate nuclear localization of heterologous proteins

The localization of the β-gal fusions was first assessed by immunofluorescence microscopy of transiently transfected HeLa cells using an anti-β-gal antibody (Promega), followed by a FITC-conjugated secondary antibody (Figure 2). β-Gal alone localized exclusively to the cytoplasm of cells and, as expected, the *lac*Z-NLS positive control was observed exclusively in the nuclei of transfected cells. The fusion containing full-length Vpx was also observed to have a predominantly nuclear phenotype. Analysis of the β-gal fusion containing partial fragments of Vpx (β-gal:61–112, β-gal:61–79) indicated that amino acids 61–79 conferred a predominantly nuclear phenotype similar to full-length Vpx. However, fusion proteins lacking this region (e.g., β-gal:1–60, β-gal:86–112) demonstrated diffuse cellular staining or only cytoplasmic localization. These findings are consistent with a previous report by Mahalingam *et al.*. demonstrating that aa 60–85 contained a transferable NLS (Mahalingam *et al.* 2001). Further dissection of this region with overlapping Vpx regions (aa 61–72, 65–75, 69–79) demonstrated that both aa 61–72 and aa 65–75 conferred a predominantly nuclear phenotype, suggesting that the overlapping region (residues 65–72) contained an NLS.

To confirm the immunofluorescence data, cells transiently transfected with the *lac*Z–*vpx* expression plasmids were fractionated and β-gal assays were performed to determine the level of activity in the nuclear and cytoplasmic compartments. Separation of the cytoplasmic and nuclear extracts was confirmed by the lack of detection of the cytoplasmic protein lactate dehydrogenase (LDH) in the nuclear fraction (data not shown). The β-gal results were expressed as a ratio of the nuclear to cytoplasmic (N/C) activity. Therefore, a ratio > 1.0 would indicate a predominantly nuclear protein. Indeed, the N/C ratio of the β-gal-NLS positive control was >7.0. The N/C ratio for β-gal alone was ~1.0 (Figure 3), which is defined as cytoplasmic in this assay. Indeed, those fusion proteins observed to be cytoplasmic by immunofluorescence all exhibited a N/C ratio of 0.84–1.43. In contrast, all fusion proteins that contained Vpx amino acid residues 65–72 ex-

hibited a N/C ratio > 2.0, confirming the results observed with the immunofluorescence. However, the ratios of the fusions that contained smaller fragments of Vpx were reduced in comparison to the full-length β-gal–Vpx fusion, suggesting other regions of Vpx may enhance nuclear localization or retention.

Alanine scanning mutagenesis of Vpx nuclear localization

Amino acids 65 to 72 are highly conserved among HIV-2 and SIV strains. This region contains several tyrosine residues and two basic residues (K^{68} and R^{70}). To further analyze the nuclear targeting potential of this region, we constructed several pEGFP-Vpx clones with alanine substitutions in this region and assessed their cellular localization in transient transfection assays (Figure 4). Alanine substitution of the two serines at positions 63 and 65 did not impair nuclear targeting of the fusion protein. However, alanine substitution of the two basic residues alone (A68,70) or together with the tyrosine residues (A66,68–71) abolished nuclear targeting of the fusion proteins, indicating that these two residues are critical for nuclear targeting of the fusion protein. These results further suggest that aa 65–72 of Vpx is an NLS. We had previously constructed several alanine-scanning mutants of Vpx in the context of a molecular clone (Jin *et al.* 2001). Two of these mutants (A74–80 and A80–86) were cloned into the GFP fusion vector and their localization was assessed by fluorescence microscopy. Both mutants produced interesting phenotypes. The mutant with alanine substitutions of aa 74 to 80 (A74–80) displayed a predominantly cytoplasmic phenotype compared to wild-type GFP-Vpx, suggesting this region is also important for nuclear localization of Vpx. Another mutant, with alanine substitutions of aa 80–86 (A80–86), while able to enter the nucleus of cells, appeared to accumulate on the nuclear membrane, suggesting a partial defect in nuclear translocation. These data are consistent with previous work from our laboratory and others that identified numerous mutations C-terminal to aa 65–72 that disrupted nuclear targeting of Vpx (Mahalingam *et al.* 2001 and Pancio *et al.* 2000).

Membrane transport and subsequent packaging of Vpx into virions require an interaction with the p6 region of Gag. To assess the membrane transport capability of each alanine scanning mutant, we cotransfected each GFP-Vpx mutant plasmid with a HIV-2 Gag-Pol plasmid (pTM-GP2) expressed by the T7-vaccinia expression system. As previously described (Pancio *et al.* 2000), GFP-Vpx was observed at the membrane of cells when coexpressed with Gag-Pol (Figure 5, arrows). Alanine substitution of aa 74–80 and 80–86 had been previously shown to be deleterious to Vpx packaging (Jin *et al.* 2001) and, as expected, both mutants failed to demonstrate membrane targeting when expressed with Gag-Pol (Figure 5). The three other alanine mutants (A63,65; A66,68–71; A68,70) exhibited membrane localization similar to wild-type GFP-Vpx, indicating that alanine substitution in this region was not deleterious to targeting of Vpx to the membrane. Furthermore, these results demonstrate that nuclear localization and membrane targeting are separable, but not discrete since A74–80 is deficient for both nuclear targeting and membrane transport.

HIV-2 ROD Vpx is not a shuttling protein

Recent studies have identified HIV-1 Vpr as a shuttling protein (Sherman *et al.* 2001). While the role of nuclear export of Vpr has not been elucidated, it has been demonstrated that shuttling is not required for packaging of Vpr, but may be important for Vpr-mediated cell-cycle arrest (Jenkins *et al.* 2001). Given the homology between Vpr and Vpx, we therefore examined whether Vpx was a shuttling protein. To determine whether Vpx shuttled, we used a heterokaryon shuttling assay developed by (Jenkins *et al.* 2001). In this assay, HeLa cells transiently transfected with GFP fusion proteins were plated onto mouse L cells and treated with polyethylene glycol (PEG) to initiate cell fusion. Cells were washed and incubated for an additional 3 h in the presence of cycloheximide. Cells were fixed and permeabilized, and nuclei were stained with DAPI. DAPI staining of L cells results in nuclei with bright, punctate, foci that are easily distinguishable from the HeLa cell nuclei (Figure 6, right panel, denoted with arrows). Shuttling can be observed under a fluorescence microscope by the movement of the GFP fusion protein to the L cell nuclei. For our studies, a GFP–HIV-1 Vpr fusion protein was

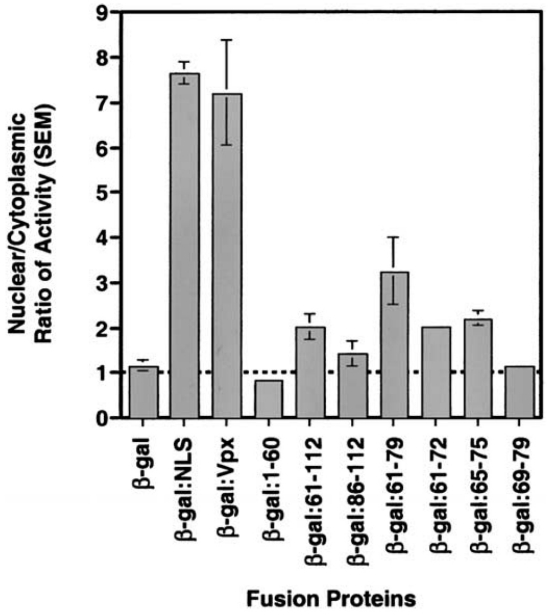

Figure 3. β-gal–Vpx fusions containing Vpx aa 65 to 72 localize primarily to the nucleus of transfected cells. 293T cells transiently transfected with the indicated expression vectors were fractionated and the β-gal activity was determined for the cytoplasmic and nuclear fractions. Separation of the cytoplasmic and nuclear fractions was monitored by detection of the cytoplasmic LDH protein in the nuclear fraction using a commercially available kit (Sigma). Results are expressed as the ratio of nuclear to cytoplasmic (N/C) activity of the β-gal–Vpx fusion proteins. Error bars denote the standard error of the mean.

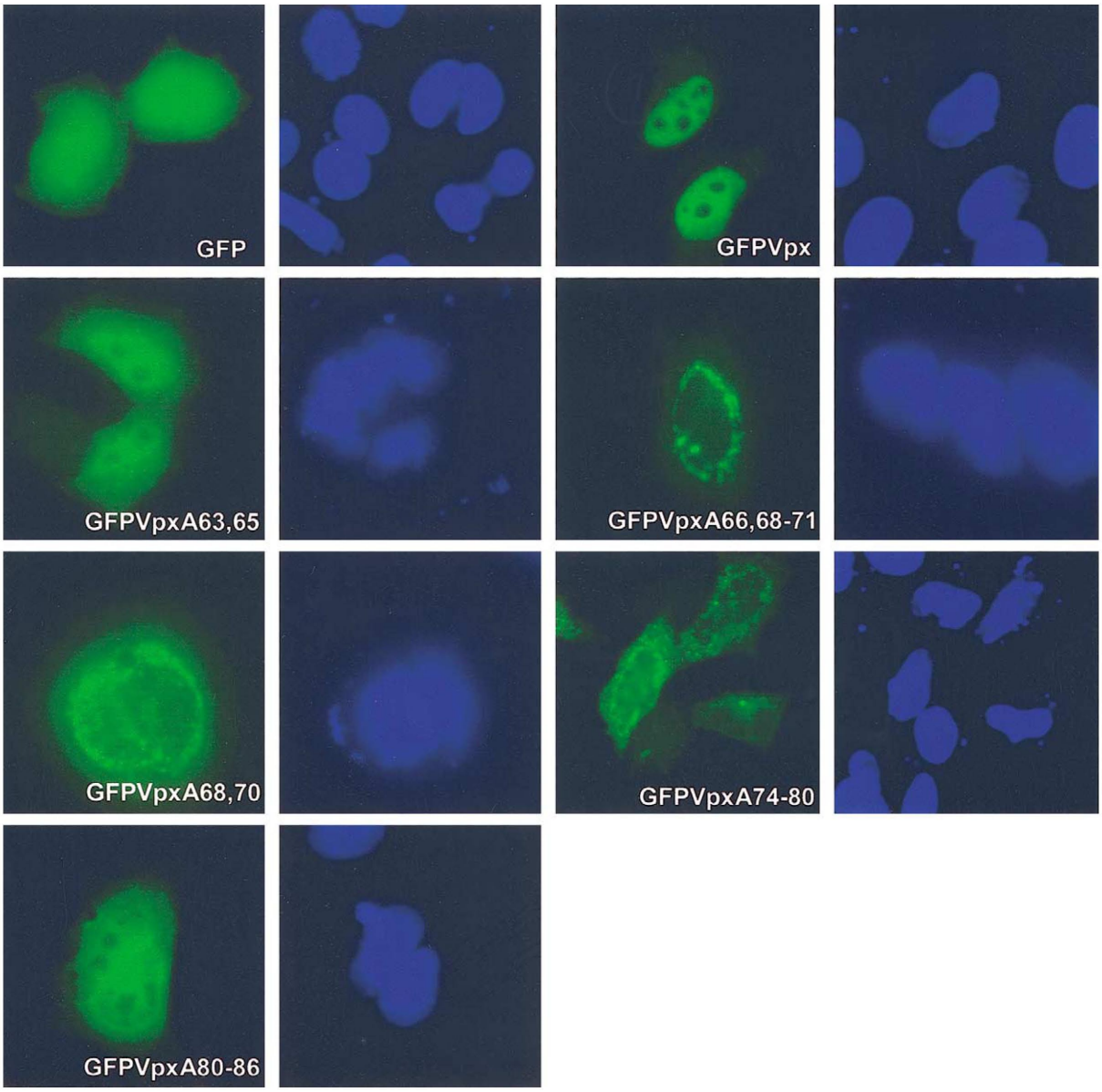

Figure 4. Alanine substitution of critical residues abolishes GFP–Vpx nuclear localization. Epifluorescence images of HeLa cells transiently transfected with GFP–Vpx fusion proteins containing the indicated alanine substitutions. After fixation, cells were permeabilized and nuclei were stained with DAPI (right panels).

used as a positive control, which was observed in the L cell nuclei (Figure 6). GFP-HIV-2 Vpx, however, did not move into the L cell nuclei when tested, indicating that shuttling did not occur. Transient transfection of GFP-HIV-2 Vpx into L cells alone produced a nuclear phenotype, confirming that the absence of GFP-HIV-2 Vpx in the L cell nuclei in the fusion assays was due to an absence of nuclear export from the HeLa cell nuclei and not a failure of nuclear import into L cells (data not shown). We also assessed whether a GFP–HIV-2 Vpr fusion protein could shuttle (Figure 6). Similar to HIV-1 Vpr, the GFP–HIV-2 Vpr protein was able to translocate from the HeLa nuclei to the L cell nuclei. The lack of shuttling of Vpx is consistent with the hypothesis that the shuttling of HIV-1 and HIV-2 Vpr is not required for virion incorporation or PIC import, but rather may be important for another function of Vpr, such as cell-cycle arrest.

Discussion

Nuclear localization of HIV-2 Vpx is necessary for the import of the viral preintegration complex into the nucleus of nondividing cells (Fletcher *et al.* 1996 and Pancio *et al.* 2000). Studies by Mahalingham *et al.*. had identified an NLS within aa 60–85 of Vpx and demonstrated that several residues within this region were critical for Vpx nuclear localization and virus replication in nondi-

viding cells (Mahalingam *et al.* 2001). The current studies have extended this work and identified a minimal transferable nuclear localization signal of HIV-2 Vpx to aa 65–72 (…SYTKYRYL…). This stretch of amino acids is highly conserved among HIV-2/SIV strains, further suggestive of the importance of this region. Classical NLSs are commonly either monopartite or bipartite. Monopartite NLSs are characterized by a single stretch of three or more basic residues, whereas bipartite NLSs are composed of two stretches of basic residues separated by approximately 10 amino acids. The Vpx NLS has two basic residues (K^{68} and R^{70}) and several tyrosines, but lacks a run of basic residues homologous to the classical monopartite NLS. Similarly, analysis of the surrounding amino acids failed to identify a motif consistent with a bipartite NLS. However, alanine substitution of K^{68} and R^{70} abolished nuclear localization of GFP-Vpx, demonstrating that the basic residues are critical components of the Vpx NLS. Therefore, given the absence of either a classical monopartite or bipartite NLS, it is likely that Vpx may require novel protein–protein interactions to move across the nuclear pore.

Although previous studies (Mahalingam *et al.* 2001 and Pancio *et al.* 2000) have demonstrated that mutations C-terminal to the NLS are deleterious to Vpx nuclear localization, we were able to map an NLS to a 10-aa region. However, the functional β-gal fractionation data would suggest that only full-length Vpx maintains complete nuclear import capability. There are several possibilities to explain this discrepancy. The most obvious explanation is that the mutations/deletions that have been previously reported may alter Vpx structure such that the NLS is compromised and no longer completely functional. A second possibility is that the C-terminal mutations disrupt another function of Vpx required for full nuclear import capability. Further studies would be needed to characterize the C-terminus of Vpx and identify any novel functional domains that affect the nuclear transport of Vpx. A third theory to explain this discrepancy could be the presence of a second NLS, such as demonstrated for HIV-1 Vpr (Jenkins *et al.* 1998). However, we feel that the broad panel of β-gal–Vpx fusions we tested would have likely identified a second NLS, if present. Finally, it could also be possible that β-gal may have compensated for a domain in Vpx to facilitate nuclear import. β-Gal may have provided a domain *in trans* to enhance a weak NLS in the smaller fusion proteins. Alternatively, since β-gal exists as a tetramer in vivo, the protein could have enhanced nuclear targeting of the smaller fusions by multiplying the effect of a single, weak NLS. Therefore, a tetramer of fusion proteins containing four weak NLSs would appear to

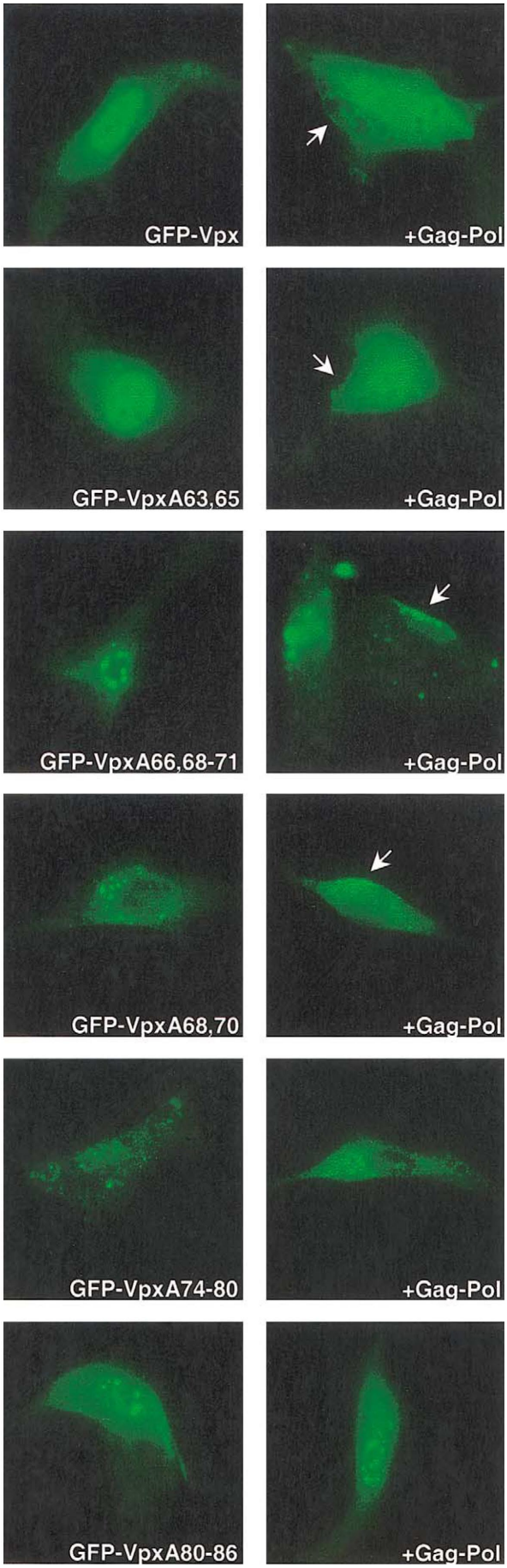

Figure 5. Membrane targeting of GFP–Vpx alanine mutants. U87 cells were infected/transfected as described under Materials and methods with the indicated GFP–Vpx expression plasmids with or without the GagPol expression plasmid pTM-GP2. Sixteen hours later cells were fixed and mounted, and images were obtained. Membrane localization is denoted by arrows.

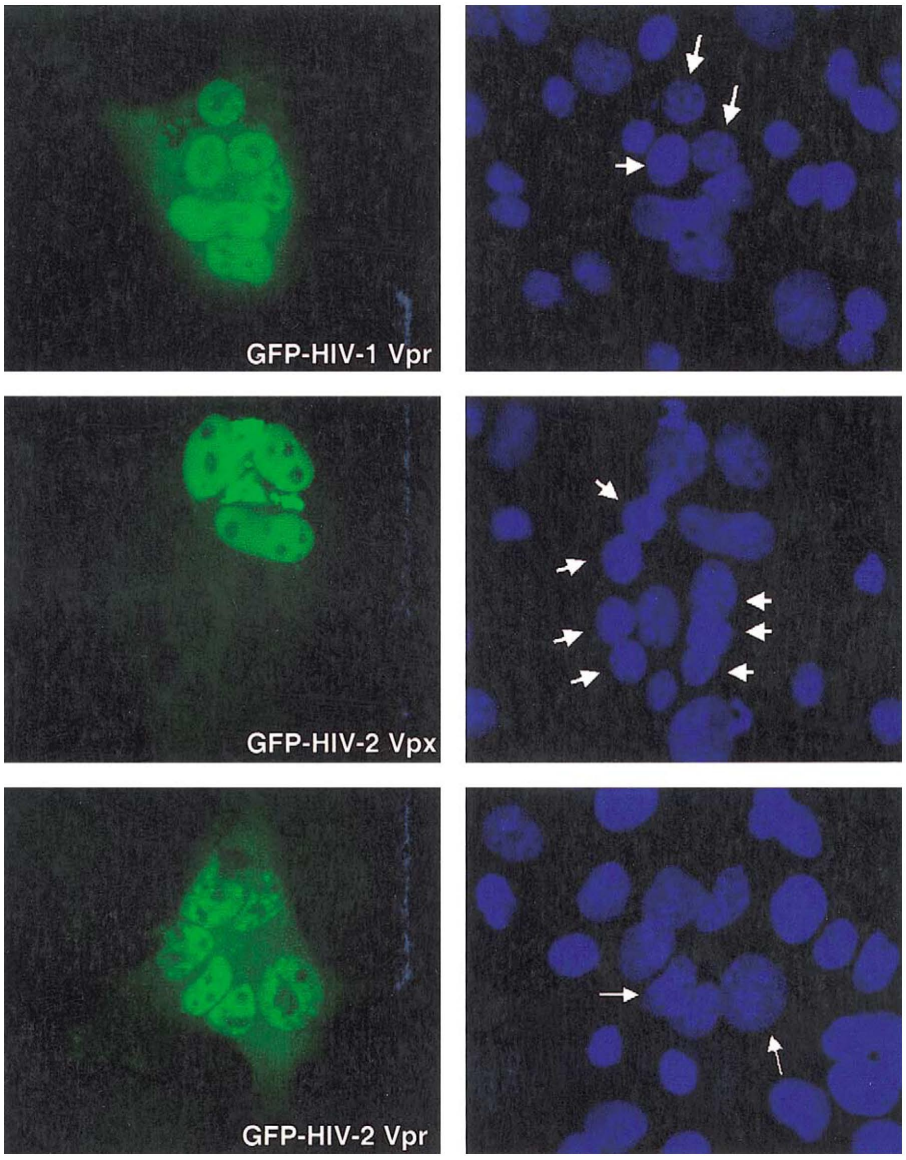

Figure 6. HIV-2 Vpx does not shuttle. HeLa cells were transiently transfected with the indicated GFP fusion plasmids 18 h prior to being plated onto L cells. After allowing the HeLa cells to settle, the cells were fused with PEG, washed, and incubated an additional 3 h in the presence of cycloheximide. Cells were fixed, permeabilized, and stained with DAPI (right panels). L cells stained with DAPI (denoted with arrows) exhibit a bright, punctate staining in comparison to HeLa cells. Shuttling is demonstrated by the movement of the GFP fusion protein into the L cell nuclei.

have stronger karyophilic properties. Nevertheless, while a larger domain of Vpx may be necessary for full function, our results demonstrate that aa 65–72 are residues critical for the karyophilic properties of HIV-2 Vpx.

In the presence of Gag, Vpx is trafficked to the plasma membrane via an interaction with p6 (Selig *et al.* 1999). Previous work demonstrated that mutation of aa 74–86 of Vpx reduced Vpx incorporation into virus-like particles and aa 80–86 were important for Vpx–Gag interactions (Jin *et al.* 2001). Consistent with these findings, alanine substitution of aa 74–80 or 80–86 abolished membrane transport of GFP-Vpx fusions. The alanine substitution of K^{68} and R^{70} abolished nuclear localization of GFP- Vpx, but had no effect on Vpx membrane transport, demonstrating that the nuclear localization and membrane transport functions are separable within Vpx. Alanine substitution of aa 74–80, however, was deleterious to both nuclear localization and membrane transport, indicating that these functional domains are not discrete. These findings support a model of Vpx transport whereby the interaction of Vpx with the p6 region of Gag suppresses the NLS of Vpx to target Vpx to the plasma membrane of cells for incorporation into virions.

The functions of HIV-1 Vpr are shared between HIV-2 Vpr and Vpx. Both proteins are packaged into virions and associate with the reverse transcription complex early after infection of cells (Fletcher *et al.* 1996); however, only Vpx is thought to play a role in PIC nuclear import (Fletcher *et al.* 1996). Recently, HIV-1 Vpr was identified as a shuttling protein (Sherman *et al.* 2001). Although the

functional significance of HIV-1 Vpr shuttling is not clear, it has been demonstrated that HIV-1 Vpr shuttling is not necessary for packaging into virions (Jenkins *et al.* 2001). Our studies indicated that HIV-2 Vpr, but not Vpx, is a shuttling protein. The inability of HIV-2 Vpx to shuttle is consistent with the idea that shuttling is not necessary for the packaging of Vpr/Vpx proteins (Jenkins *et al.* 2001). Furthermore, the inability of Vpx to shuttle suggests that shuttling plays no role in the nuclear import of the HIV-2 PIC. Rather, since HIV-2 Vpr shuttles, it would seem likely that shuttling is important for Vpr-mediated G2 cell-cycle arrest or another undefined function.

We have identified a minimal NLS of HIV-2 Vpx; however, questions still remain to define the exact role of Vpx in PIC nuclear import. The lack of a "classical" NLS suggests that Vpx may use a novel pathway to access the nuclei of cells. Future studies to identify cellular proteins that interact with Vpx will further define the pathway through which Vpx accesses the nucleus and the role the Vpx protein plays as a component of the HIV-2 PIC. Delineation of the role the Vpx/Vpr proteins play in the nuclear import of the PIC may lead to the development of novel therapeutic interventions of HIV infection.

Materials and methods

Plasmid construction

Plasmids pTM-GP2 and pEGFPVpx have been previously described (Horton *et al.* 1994 and Pancio *et al.* 2000). To construct *lac*Z fusions, the *lac*Z ORF was PCR amplified from pCH110 (Amersham Biosciences, Piscataway, NJ) using Redi-Taq as described by the manufacturer (Sigma-Aldrich, St. Louis, MO). Primer sequences are available upon request. The 3′ primer in the reaction contained an artificial *Xho*I restriction site inserted immediately 5′ of the stop codon. The PCR product was TA cloned into pcDNA3.1/V5-His-TOPO as described by the manufacturer (Invitrogen, Carlsbad, CA). Positive clones (pclacZ(*Xho*I)) were identified by restriction mapping and sequencing the 5′ and 3′ insert junctions. All *vpx* inserts for fusion vectors were cloned into the *Xho*I site of the plasmid. For full-length and larger segments of *vpx*, regions were PCR amplified with HIV-2 ROD-specific PCR primers containing *Xho*I sites on both 5′ and 3′ primers, with premature stop codons in the 3′ primers of truncated fragments. For regions of 12 aa or less, overlapping primers were synthesized (IDT Technologies, Iowa City, IA), annealed, phosphorylated, and ligated into digested pclacZ(*Xho*I). All clones were confirmed by sequence analysis. Alanine substitutions were inserted into pEGFP-Vpx by Excite Mutagenesis (Stratagene, La Jolla, CA) as described by the manufacturer using primers with site-specific substitutions. Alanine substitutions were confirmed by sequence analysis.

Fractionation assays

293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin/streptomycin. Cells were transfected using TransIT-LT1 reagent with 2–4 μg of indicated *lac*Z-fusion plasmids as described by the manufacturer (PanVera, Madison, WI). Forty-eight hours posttransfection, cells were harvested by treatment with PBS + 5 mM EDTA and transferred to 1.5 mL microcentrifuge tubes. Nuclear and cytoplasmic fractions were obtained using a commercially available kit (Pierce Chemical Co., Rockford, IL). Separation of the fractions was confirmed by lack of detectable LDH enzyme in the nuclear fractions using a commercially available kit (Sigma-Aldrich). Protein concentrations of each fraction were determined using a commercially available kit (Pierce Chemical Co.). The enzymatic activity of the β-gal fusion was determined using a colorimetric microtiter plate assay. Results are presented as a ratio of the nuclear to cytoplasmic β-gal activity. The results for each fusion represent at least three independent experiments, with three separate transfections per experiment.

Immunofluorescence analysis

HeLa cells (5×10^5) seeded onto glass coverslips were transfected with 2 μg of *lac*Z fusion expression plasmids as described above and incubated overnight. Cells were washed, fixed with 4% paraformaldehyde in PBS, washed again, and then permeabilized with 0.2% Triton X-100 in PBS for 5 min. β-Gal was detected using a monoclonal antibody (Promega), followed by a FITC-conjugated anti-mouse IgG secondary antibody (Sigma). After the antibody incubations, the cells were incubated for 5 min with 2 μg/mL DAPI to allow for visualization of nuclei. Cells were visualized and images obtained using a fluorescence microscope equipped with an Optronics Magnafire imaging system (Optronics, Goleta, CA). Multiple images were obtained from at least two independent transfections and images presented in figures are representative of the dominant phenotype observed.

GFP-Vpx mutant localization and membrane transport assays

U87 cells maintained in DMEM supplemented with 15% FBS and penicillin/streptomycin were plated onto glass coverslips in six-well plates. For localization assays, HeLa cells were transfected with 2 μg of indicated expression plasmid as described above. For membrane transport assays, U87 cells were infected with VPT7-3 vaccinia virus for 1 h in OptiMEM (Invitrogen) and then transfected in normal media with 2 μg of indicated GFP-Vpx mutant or 1 μg GFP-Vpx mutant, and 1 μg pTM-GP2. U87 cells were grown overnight, fixed with 4% paraformaldehyde in PBS, washed 3× in PBS + 1% FBS, and mounted onto slides. For localization studies, HeLa cells were permeabilized with 0.2% Triton X-100 for 5 min after fixation and washed, and nuclei were stained with 2 μg/mL DAPI and washed again prior to mounting. Slides were visualized and multiple images obtained as described above. Images presented in figures are representative of the dominant phenotype for each mutant.

Heterokaryon shuttling assays

HeLa and L cells were maintained as described above. HeLa cells (5×10^5) were seeded into one well of a six-well plate and the next day were transfected with 2 μg of GFP-fusion plasmid. That same day, L cells were seeded onto coverslips in six-well plates at a density of 5×10^5 cells/well. The next day, the HeLa cells were trypsinized and plated onto the L cells. After allowing the HeLa cells to settle (3–5 h), the coverslips were removed and inverted onto 50% w/v PEG (Sigma-Aldrich) for 2 min to initiate cell–cell fusion. The cells were then washed with PBS and incubated for an additional 2 h in media containing 50 μg/mL cycloheximide. Cells were fixed, permeabilized, stained with DAPI, and mounted, and images were obtained as described above.

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