HIV-2 viral protein X (Vpx) ubiquitination is dispensable for ubiquitin ligase interaction and effects on macrophage infection

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ABSTRACT

HIV-2 Vpx, a virus-associated accessory protein, is critical for infection of non-dividing myeloid cells. To understand the function of Vpx ubiquitination, interaction with an E3 ubiquitin ligase complex, and ability to overcome an inhibition of reverse transcription, we analyzed Vpx lysine mutants for their function and replication capability in macrophages. Both WT Vpx and Vpx TA (lysine-less Vpx) localized to the cytoplasm and nucleus in HeLa cells. All HIV-2 Vpx lysine mutants were functional in virion packaging. However, ubiquitination was absent with Vpx TA and Vpx K84A mutants, indicating a lack of ubiquitin on positions K68 and K77. Mutants Vpx K68A and K77A were unable to infect macrophages due to impaired reverse transcription from loss of interaction with the ubiquitin substrate receptor, DCAF1. Even though Vpx K84A lacked ubiquitination, it bound DCAF1, and infected macrophages comparable to WT Vpx.

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Introduction

HIV infects non-dividing cells such as macrophages and dendritic cells (DCs). Once infected, these cells help to propagate and disseminate the virus throughout the host (Hirsch et al., 1998). The viral life cycle requires reverse transcription and transportation of the viral genetic material to the nucleus, as part of the pre-integration complex (PIC). The mechanics of cyto-nuclear translocation of the PIC have not been delineated although several viral proteins have been implicated in this process, including integrase (IN), matrix (MA), viral protein R (Vpr) for HIV-1, and viral protein X (Vpx) for HIV-2/SIV (Depienne et al., 2000).

All HIV/SIV strains encode for Vpr, but members of HIV-2/SIVmAd/SIVmac also encode for Vpx, a 12–16 kDa virion-associated accessory protein that is essential for early viral replication in macrophages (Guyader et al., 1989; Hirsch et al., 1998; Lu et al., 1993). The sequence of Vpx is similar to that of Vpr, and the vpx gene was proposed to have arisen through a gene-duplication event (Tristem et al., 1990). Nevertheless, the functions of Vpr and Vpx are distinct. Vpr, but not Vpx, induces cell cycle arrest and apoptosis (Belzile et al., 2007; Fletcher et al., 1996). Vpx, however, promotes reverse transcription and nuclear import of viral PICs in non-dividing cells (Belshan et al., 2006; Fujita et al., 2008; Goujon et al., 2007; Hirsch et al., 1998). Both, Vpr and Vpx are incorporated into virions in quantities comparable to the viral Gag protein, although one study points to a smaller ratio of Vpr to Gag in virions (Kewalramani and Emerman, 1996; Muller et al., 2000).

Viruses modulate cells by hijacking cellular complexes and pathways (Fujimura et al., 2007; Goff, 2007). One of the cellular systems that is hijacked is the ubiquitin proteasome system (UPS), which is responsible for ubiquitination and degradation of proteins (Horvath, 2004; Leupin et al., 2005; Margottin et al., 1998; Mehle et al., 2004). Ubiquitination is a post-translational modification of proteins that not only regulates the steady-state levels of proteins, but also regulates other functions, including transcription and cyto-nuclear translocation (Hershko and Ciechanover, 1998). In conjunction with the ubiquitin-activating E1 and E2 proteins, ubiquitin E3 ligases conjugate ubiquitin to lysine residues present in substrates. Cullin4A-RING E3 ubiquitin ligase complex, composed of the cullin4A (CUL4A) scaffold protein, damaged DNA binding protein 1 (DDB1) adaptor, and DDB1 and CUL4A-associated factor 1 (DCAF1) substrate receptor is commandeered by Vpr to cause G2 arrest (Angers et al., 2006; Belzile et al., 2010; Belzile et al., 2007; Higa et al., 2006; Le Rouzic et al., 2007; Zhao et al., 1994). Vpx also interacts with the CUL4A–DDB1–DCAF1 complex, but instead of causing G2 arrest, Vpx is thought to direct ubiquitination and degradation of a restriction factor that has recently been identified as SAMHD1 (Hrecka et al., 2011; Laquette et al., 2011; Le Rouzic et al., 2007; Wen et al., 2007). The SAMHD1 mechanism of restriction is not fully understood as it fails to restrict viral infection in...
Confocal microscopy revealed that Vpx Wt was localized to both the cytoplasm and nucleus (0 cells had Vpx in a perinuclear aggregate), and 2 cells had Vpx localized to the cytoplasm, and 17 were nuclear. Out of 100 cells expressing Vpx TA, 78 cells showed Vpx localized to the cytoplasm and nucleus (23 cells had perinuclear aggregation), 6 cells had Vpx localized to the cytoplasm, and 16 cells had Vpx with nuclear localization. There was little difference in the localization pattern between the Vpx mutants with single lysine substitutions (data not shown). Even though the Vpx TA mutant had a similar pattern of cytoplasmic and nuclear localization compared to Vpx Wt, 23% of those cells had a phenotype in which Vpx TA aggregated around the nucleus (characteristic aggregate photo of Vpx TA is shown in Fig. 2).

**Localization of Vpx lysine mutants**

To determine the cellular localization of Vpx lysine mutants, we transiently transfected HeLa cells that were seeded on coverslips. Confocal microscopy revealed that Vpx Wt was localized to both the cytoplasm and the nucleus in approximately 80% of cells (Fig. 2).

![Fig. 1. Schematic representation of the 6xHis-tagged HIV-2 Vpx constructs and their expression.](image)

**Results**

Many vpx mutations have been made in order to study Vpx function, however, few studies examined the importance of Vpx lysine residues, and only one manuscript reported on ubiquitination of Vpx from SIV (Sharova et al., 2008). To determine the effect of lysine substitutions on Vpx function, we engineered lysine-to-alanine substitutions in Vpx from the HIV-2 GH-1 isolate. Single Vpx substitutions were made at lysine positions 68, 77, and 84, and a triple substitution, designated TA (triple alanine), was made in all three lysines (Fig. 1). All Vpx mutants were fused to a 6xHis tag at the N-terminus. None of the lysine substitutions hindered Vpx expression in 293T cells (Fig. 1).

Out of a 100 cells expressing Vpx Wt, 81 cells showed Vpx localized to the cytoplasm and nucleus (0 cells had Vpx in a perinuclear aggregate), and 2 cells had Vpx localized to the cytoplasm, and 17 were nuclear.

**UBIQUITINATION OF HIV-2 Vpx USING K48R UBQUITIN MUTANT**

Several studies demonstrated that Vpr and Vpx take advantage of the cellular ubiquitin machinery by hijacking the CUL4A–DDB1–DCAF1 E3 ubiquitin ligase complex to degrade a viral restriction factor (Bergamaschi et al., 2009; Le Rouzic et al., 2007; Sharova et al., 2008). Since Vpx plays a role in commandeering the cellular ubiquitin system, we wanted to determine the ubiquitination status of HIV-2 Vpx mutants.

In our study, we show that Vpx is post-translationally modified, as seen by the presence of higher molecular bands detected with an anti-Vpx mAb in lysates of cells transfected with 6xHis-tagged Vpx Wt (Fig. 4A). To determine the ubiquitination status of HIV-2 Vpx, and to examine the importance of ubiquitinated Vpx, the 6xHis-tagged Vpx lysine mutants were transfected into 293T cells in conjunction with a plasmid that encodes a Flag-tagged ubiquitin K48R mutant (Ward et al., 1995). The ubiquitin K48R, which is deficient in the formation of polyubiquitin chains at position K48, and thus lacks the ability to tag substrates for proteasomal degradation, was used to enrich Vpx that is ubiquitin-modified. The bands detected with anti-Flag mAb from purified Vpx reactions indicate that Wt Vpx is ubiquitinated (Fig. 4B). The 23 kDa band represents ubiquitination of Wt, K68A, and K77A Vpx mutants, but it is not present with K84A Vpx or TA Vpx mutant. This is also found when the membrane is blotted with mAb to Vpx (Fig. 4C). These findings suggest that position K84 is the primary attachment site for ubiquitin. Several higher molecular weight bands that are observed with all Vpx mutants are presumably ubiquitinated cellular proteins that interact with Vpx and proteins that non-specifically bind to Ni-NTA beads (compare lane with FlagUbK48R to lane with Vpx Wt/FlagUbK48R) (Fig. 4B).

The same membrane blotted with the anti-Vpx mAb provides a better comparison of endogenous and exogenous ubiquitination (Fig. 4C). The band pattern observed with anti-Vpx mAb is similar...
to that observed with the anti-Flag mAb (comparing Figs. 4B and C). The 23 kDa band is present in lanes with Vpx Wt, K68A, and K77A, representing Vpx that is ubiquitin-modified, however this 23 kDa band is absent with the TA and K84A Vpx. Endogenous ubiquitin modification of Vpx can be observed in the same lanes, represented by bands with a slight faster electrophoretic mobility than that of FlagUbK48R-Vpx. More than 4 bands representing the endogenous ubiquitin modification are displayed in Vpx Wt, and in Wt/FlagUbK48R even though this HIV-2 Vpx has only three lysines. This is due to the fact that endogenous ubiquitin is able to form poly-ubiquitin chains while UbK48R does not. Endogenous polyubiquitination is also evident in Vpx K68A and K77A lanes. Vpx K84A lacks ubiquitin modification and is similar to Vpx TA.

Ubiquitination has also been reported to occur on non-lysine residues: cysteines, serines, and threonines (Cadwell and Coscoy, 2005; Herr et al., 2009; Tait et al., 2007). Bonds between ubiquitin and non-lysine residues are susceptible to alkaline environment due to the ester bond formation (Wang et al., 2007). The amide bond between ubiquitin and the lysine residue is more resistant to alkaline treatment. Although our Vpx TA mutant had no evident ubiquitination, we wanted to confirm that Vpx Wt was ubiquitin-modified on lysine residues. Cells that were transfected with 6xHis-tagged Vpx Wt and Vpx TA were lysed in denaturing conditions and boiled to dissociate Vpx-interacting proteins. After Vpx purification, the lysates were halved. One half was treated with PBS and the other half was treated with 0.1 M NaOH. Ubiquitination of Vpx was not disturbed.

Fig. 2. Vpx exhibits a perinuclear localization pattern in HeLa cells. The 6xHis-tagged Vpx Wt and 6xHis-tagged Vpx TA constructs were transfected into HeLa cells cultured on coverslips. The cells were fixed, permeabilized, and stained with anti-Vpx and anti-Nup98. Secondary antibodies included Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 562-conjugated goat anti-rabbit IgG. Nuclei were visualized with TO-PRO3 stain. Confocal microscopy was used to obtain images at 24 h post-transfection.
**Viral Incorporation of HIV-2 Vpx WT and Vpx lysine mutants.** Immunoblot analysis of Vpx mutant proteins in 293T cellular lysates and virions. HEX 293T cells were co-transfected with proviral clone pES or pESdelVpx (which lacks Vpx expression) and 6xHis-tagged Vpx WT or mutant constructs. On day three post-transfection, supernatants were clarified through a 0.22 μm filter, and virus concentrated on a 20% sucrose cushion. Cellular and viral lysates were resolved by SDS-PAGE. Both, virus and cell lysates were analyzed by Western blot with an anti-Vpx mAb and HIV-1 antiserum against Gag. Band intensity was determined by imaging (BioRad Image Lab 3.0). Relative intensity was normalized to that of capsid (CA) levels, relative to those of pESdelX+Wt. Relative density is reported as ratios of supernatant to cellular lysates.

**Macrophage infection with HIV-2 Vpx lysine mutants**

It is well established that in the absence of Vpx, HIV-2 fails to produce infect macrophages (Fletcher et al., 1996; Ueno et al., 2003; Wolfrum et al., 2007; Yu et al., 1991). A previous study suggested that ubiquitination is important for Vpx to promote productive infection of macrophages (Sharova et al., 2008). Thus, we evaluated the endogenous polyubiquitination of Vpx with the alkaline treatment (Wt Vpx ubiquitination is reported as ratios of supernatant to cellular lysates.

**Lysine mutations and Vpx interaction with DCAF1**

Lack of interaction between DCAF1 and Vpx is detrimental to replication of HIV-2 and SIV in MDMs due to impairment of reverse transcription (Bergamaschi et al., 2009; Le Rouzic et al., 2007; Srivastava et al., 2008). Based on Vpr mutations, DCAF1 interaction with Vpx was delineated to position Q76 in Vpx (Le Rouzic et al., 2007). However, Bergamaschi et al., 2009 reported that mutation at position K77 in Vpx also interrupts Vpx-DCAF1 binding. Therefore, we examined whether Vpx lysine mutations affect the interaction between DCAF1 and Vpx, in order to determine if Vpx ubiquitination is essential for binding to E3 ubiquitin ligase complex. Vpx lysine substitution at positions K68, K77, and TA diminished the interaction between DCAF1 and Vpx (Fig. 7). In contrast, Vpx K84A interacted with DCAF1 comparably to WT Vpx.

**Discussion**

Vpx lysine residues are located in regions previously shown to be important in viral replication. Residue K68 is situated in a nuclear localization signal thought to be important for the nuclear import of PICs (Belshan and Ratner, 2003; Belshan et al., 2006). Residue K77 is adjacent to Q76, which is critical for DCAF1 interaction and, as a result, binding to an E3 ligase (Le Rouzic et al., 2007). Both residues, K68 and K77, are highly conserved among HIV-2 and SIV strains, while position 84 is primarily either a K or an R, depending on the viral isolate (Mahnke et al., 2006). Conservation of the lysines and regions where they are located indicates their importance in Vpx function and viral replication.

With our Vpx lysine mutants, we found that while Vpx WT was ubiquitinated, marked by the presence of ubiquitin bands detected with Flag mAb, Vpx TA mutant lost all ubiquitination, as did the Vpx K84A mutant. We observed that our HIV-2 Vpx K84A mutant lacked ubiquitination even though other lysine residues were present in Vpx, at positions 68 and 77. These results are consistent with the results reported by Sharova and colleagues, in which SIV Vpx K84R mutant had the greatest defect in ubiquitination (Sharova et al., 2008).

When the same membrane was blotted with antibody to Vpx, a clearer picture of Vpx ubiquitination emerged (Fig. 4C). Wild-type Vpx alone displayed polyubiquitin modification made by endogenous ubiquitin while Vpx WT, in conjunction with exogenous ubiquitin, showed a single band at 23 kDa due to monoubiquitination by the FlagUbK48R mutant. Several bands corresponding to polyubiquitination by the endogenous enzyme were also evident. A comparable pattern appeared with Vpx K68A and K77A, while Vpx K84A and TA had no evidence of modification by either endogenous or exogenous ubiquitin. These results support our conclusion that position K84 in Vpx is the primary site for ubiquitination, and that Vpx WT is polyubiquitinated.

It is unclear if ubiquitination of Vpx is a result of binding to 1) SAMHD1, 2) DCAF1 and the Cu4 E3 machinery, or 3) a different ubiquitin ligase complex. HIV-1 Vpu has been shown not only to serve as an adaptor for CD4 degradation, but also as a substrate for an E3 ligase (Belaidouni et al., 2007). An accessory protein Vif from HIV-1 serves as an adaptor that brings in the APOBEC3F substrate for ubiquitination by the Cu5 E3 ligase and as a substrate that is ubiquitinated (Liu et al., 2005).
The subcellular localization of Vpx was reported to be predominantly nuclear. However, Vpx exhibits perinuclear and cytoplasmic localization in a small percentage of cells (Goujon et al., 2007; Kappes et al., 1993; Mahalingam et al., 2001; Mahnke et al., 2006; Mueller et al., 2004; Pancio et al., 2000). Wild-type Vpx localized mainly to the cytoplasm and nucleus (Fig. 2). Vpx TA had more of an aggregated perinuclear and cytoplasmic distribution. The aggregated phenotype observed with Vpx TA mutant may be attributed to the triple lysine substitution, which could cause protein destabilization. However, this Vpx mutant was still functional in virus particle packaging.

After macrophage infection, early and late reverse transcription products for virions with trans-complemented Wt Vpx were at comparable levels to that of Vpx incorporation generated by the fully functional ES HIV-2. However, virions with Vpx TA, K68A, and K77A mutants had severely impaired early and late reverse transcription products compared to the products obtained with ES or with Vpx Wt virions, indicating that mutations at positions K68 and K77 are detrimental to reverse transcription. Interestingly, Vpx mutation K84A had no effect on early or late reverse transcription product levels and the levels were similar to those observed with Vpx Wt and ES, indicating that Vpx ubiquitination is dispensable for replication in MDMs. Sharova et al., 2008, reported that SIV VpxM2 (K68, 77R mutation) was ubiquitinated but manifested a defect in infectivity. This would indicate that either ubiquitination of K84/K85 or presence of the amino acid K68 or K77 is essential for replication in MDMs. The lack of MDM infection with Vpx that has a substitution at position K77 can be explained by the lack of interaction with DCAF1 (Bergamaschi et al., 2009). Also, our results demonstrate that Vpx K68A mutant has severely decreased binding with DCAF1, whereas, the Vpx K84A mutant interacts with DCAF1. These results mirror the results obtained with the macrophage infections. Overall, we demonstrate a novel Vpx residue, K68, that is critical for Vpx–DCAF1 interaction. Thus far, mutations of residues K68, Q76, K77, and F80 (SIV Vpx) affect Vpx binding to DCAF1 and consequently block viral infection. In addition, we show that absence of ubiquitination on residue K84 of Vpx is dispensable for MDM infection.

**Materials and methods**

**Tissue culture, plasmids, and virus**

HeLa, 293T, and TZM-bl cells were maintained in DMEM with 10% FBS, 1 mM Na-pyruvate and 100 µg/ml penicillin-streptomycin.
Fig. 5. Alkaline treatment of Vpx. (A) HEK 293T cells were transfected with 6xHis-tagged Vpx construct and pcDNA (mock). Three days post-transfection, the cells were lysed in denaturing conditions, and Vpx was purified using Ni-NTA beads. After several washes with lysis buffer, Vpx bound to beads was divided into two samples. One sample was treated with PBS (control) and the other sample was treated with 0.1 M NaOH. Both parts were incubated for 2 h at 37 °C. Proteins were resolved by SDS-PAGE and analyzed by Western blot using an anti-Flag mAb. (B) Endogenous ubiquitination was detected by re-blotting the PBS and NaOH treated blot with anti-Vpx mAb.

Fig. 6. Effect of Vpx lysine substitutions on viral infectivity. Viral DNA synthesis in human MDMs. The VSV-g pseudotyped cell-free virus was prepared in 293T cells that were co-transfected with proviral construct pES, and pESdelX (lacks expression of Vpx), and 6xHis-tagged Vpx mutant constructs and pVSV-g. The MDMs were mock infected, infected with viruses expressed from pES, or pESdelX (which lacks Vpx expression), or viruses expressed from pESdelX trans-complemented with 6xHis-tagged Vpx lysine mutants. Equal amounts of virus, which was titered on TMZ-bl reporter cells, were used in the infection assays, and all viral samples were treated with DNase I before infection of MDMs. AZT (10 μM) was added as a control 24 h and 6 h prior to infection to specified wells. Cellular DNA was extracted at 24 h and 48 h post-infection and subjected to real-time PCR analysis of early and late reverse transcription products. The data are an average of two independent experiments with error bars representing the standard deviation of the two samples.
Monocyte derived macrophages (MDMs) were maintained in Iscoves Modified Dulbeccos Medium (IMDM), 10% human AB serum, 100 μg/ml penicillin-streptomycin, 4 mM l-glutamine, and 500 U/ml of recombinant human GM-CSF. MDMs were isolated from peripheral blood of healthy donors via elutriation.

HIV-2 GH-1 Vpx was expressed as a 6xHis fusion protein (N-terminus) from a pRBG4 vector (Lee et al., 1991). The GH-1 Vpx was PCR amplified to include 6xHis tag flanked by EcoRI and Clal restriction enzyme sites and cloned into pRBG4 that was digested with EcoRI and Clal. All lysine to alanine substitutions were generated with the QuickChange method (Stratagene, La Jolla, CA). The resulting Vpx lysine plasmids were pRBG4-6xHis-VpxWt, pRBG4-6xHis-VpxTA (lysine-less), pRBG4-6xHis-VpxK68A, pRBG4-6xHis-VpxK77A, and pRBG4-6xHis-VpxK84A. The ubiquitin encoding plasmid, CMV2-Flag-UbK48R, was engineered by PCR amplification of K48R ubiquitin to include flanking NotI and EcoRI restriction sites and ligated into the N-terminal Flag expression vector pFlag-CMV-2 (Sigma, St. Louis, MO). All plasmid constructs were verified by restriction digestion and sequencing. The HIV-2 functional proviral clones pES and pESdelX were used for transfection of 293T cells that were seeded 24 h pre-transfection on coverslips. pRBG4 was used as mock control. Twenty four hours post-transfection, cells were lysed and Vpx was immunoprecipitated using anti-Vpx mAb and Protein A agarose beads. Lysates were resolved with SDS-PAGE and immunoblotted for DCAF1 and Vpx.

Fig. 7. Vpx lysine substitutions affect the interaction of Vpx with DCAF. HEK 293T cells were transfected with mock, 6xHis-tagged Vpx Wt, 6xHis-tagged Vpx TA, 6xHis-tagged Vpx K68A, 6xHis-tagged Vpx K77A, and 6xHis-tagged Vpx K84A. Forty eight hours post-transfection, the cells were lysed, and Vpx was immunoprecipitated using anti-Vpx mAb and Protein A agarose beads. Lysates were resolved with SDS-PAGE and immunoblotted for DCAF and Vpx.

MDM infection and real-time PCR

Seventy two hours post-transfection, supernatants were clarified through a 0.22 micron filter and viral titers were assessed on TZM-bl reporter cells (Derdeyn et al., 2000; Platt et al., 1998; Wei et al., 2002). TZM-bl cells were seeded 24 h pre-infection in a 96 well plate. Supernatants were diluted 1:5 in DMEM supplemented with 2% FBS, and subsequently with 4 sequential 1:5 dilutions. The cells were incubated with the viral dilutions for 2 h in a 37 °C incubator supplemented with 5% CO2. After 2 h, DMEM with 10% FBS was added to each well, and the cells were incubated for additional 48 h. To determine luciferase activity, cells were lysed and 10 μl of each sample was transferred to a luciferase tube. Reporter Lysin Buffer (Promega, Madison, WI) was injected and light intensity was measured using an Optocompl luminometer. Uninfected cells represented the background luciferase activity, which was subtracted from all other samples.

MDMs were allowed to differentiate for a week before equal amounts of virus were added for infection. All viruses were treated with Turbo DNase (20 U/ml) (Ambion) for 45 min at 37 °C. Cells were cultured for 24 h and 48 h after infection, at which point DNA was collected for detection of reverse transcription products. Macrophage DNA was isolated using DNeasy tissue kit (Qiagen). Real-time PCR (iCycler; BioRad) was used to detect early and late reverse transcription products using iQ Supermix reagent (BioRad) and primers for early US and late gag gene reverse transcription products. Standards were made using proviral plasmid pES.

Localization in HeLa cells

The pRBG4-6xHis-VpxWT and pRBG4-6xHis-VpxTA plasmids were used for transfection of HeLa cells that were seeded 24 h pre-transfection on coverslips. pRBG4 was used as mock control. Twenty four hours post-transfection, cells were washed with 1 x PBS, fixed with 2% formaldehyde, washed, permeabilized with 0.2% Triton X-100 in PBS for 6 min, and stained with anti-Vpx mAb and anti-
Nup98 pAb in 1XPBS/5%BSA for 1 h. After a wash step, the cells were stained with Alexa Fluor-488 (mouse) and Alexa Fluor-562 (rabbit) (Molecular Probes) for 15 min in the dark. Nuclei were counterstained with TO-PRO3 (Invitrogen). Cells were visualized using LSM-510 META Laser Scanning Confocal Microscope.

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