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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.

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/SIV_{SM}/SIV_{MAC} 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 (Fujimuro 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

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undifferentiated THP-1 cells and HEK 293T cells, although endogenously expressed in these cells (Hrecka et al., 2011). The siRNA knock down of DCAF1 inhibits reverse transcription in macrophages, highlighting the importance of Vpx–DCAF1 interaction in viral replication (Bergamaschi et al., 2009; Fletcher et al., 1996; Fujita et al., 2008; Goujon et al., 2007; Srivastava et al., 2008). Mutation of residue Q76 in Vpx, which disrupts Vpx–DCAF1 interaction, also results in an HIV-2 growth defect in macrophages (Bergamaschi et al., 2009; Le Rouzic et al., 2007).

SIV Vpx was shown to be modified by ubiquitin and a suggestion was made that Vpx is ubiquitinated on residues other than lysine (Sharova et al., 2008). This study suggested that lack of Vpx ubiquitination led to decreased macrophage infection, and no association with DDB1. Ubiquitin addition to non-lysine residues is not new but so far it is found infrequently. Wang and colleagues have shown that serines and threonines can be modified with ubiquitin on MHC-I and Tokarev et al. indicated that Vpu leads to ubiquitination of serine/threonine on BST-2 to induce its down-regulation (Tokarev et al., 2011; Wang et al., 2007). The objective of this study was to analyze the effects of lysine substitutions in HIV-2 Vpx on ubiquitination, and function of Vpx lysine mutants in macrophage infection. We show that mutation of all three lysines or individual lysine residues in HIV-2 Vpx does not affect Vpx expression or incorporation into HIV-2 virions. In this study, we show ubiquitination only on K84. However, K84 ubiquitination is dispensable for DCAF1 interaction and macrophage infection.

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).

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).

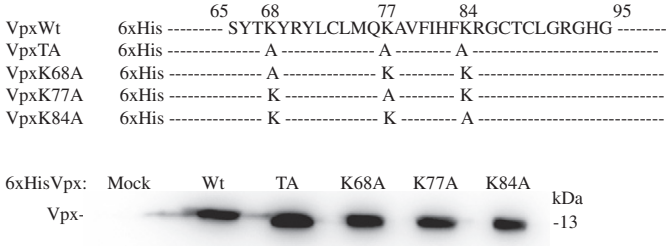

Fig. 1. Schematic representation of the 6xHis-tagged HIV-2 Vpx constructs and their expression. Schematic depiction of Vpx lysine residues that were individually substituted for alanine at positions 68, 77, 84 and in triple combination, TA (triple alanine), in which case every lysine in Vpx is substituted. All constructs were N-terminally labeled with 6xHis tag. Only partial amino acid sequences are shown for Vpx. Western blot showing the expression of the 6xHis-tagged Vpx mutants. HEK 293T cells were transfected with mock, 6xHis-tagged Vpx wild-type (Wt), 6xHis-tagged Vpx triple alanine (TA), 6xHis-tagged Vpx K68A, 6xHis-tagged Vpx K77A, and 6xHis-tagged Vpx K84A constructs. Forty eight hours post-transfection, cells were lysed and proteins were resolved by SDS-PAGE. Blots were analyzed for Vpx expression using an anti-Vpx mAb.

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 aggregation), 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).

Packaging of Vpx lysine mutants into virus particles

Vpx incorporation has been delineated to the interaction between p6 of Gag and Vpx residues Leu74 and Ile75, although some mutations, substitution of other residues at positions 73–89 to alanines, decrease incorporation of Vpx into virions (Jin et al., 2001; Rajendra Kumar et al., 2005). To determine if the Vpx lysine substitutions affect incorporation of Vpx into HIV-2 virions, we *trans* complemented the HIV-2 proviral clone (pESdelX) that has an impaired *vpx* coding region with plasmids encoding the Vpx lysine mutants. For this purpose, transient co-transfection of 293T cells with pESdelX and 6xHis-tagged Vpx lysine mutants was carried out. All of the Vpx lysine mutants were incorporated into HIV-2 virions, and none of the Vpx lysine substitutions, individually or in the triple combination, had a significant effect on the efficiency of Vpx packaging into virus particles (Fig. 3). Absence of a Vpx band in pESdelX lane is due to mutations in *vpx* at the initiating and first internal methionine, plus a frameshift that introduces a termination codon at position 70 (Hu et al., 1989).

Ubiquitination of HIV-2 Vpx using K48R ubiquitin 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 presumed to be 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