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HIV-1 Vpr suppresses the cytomegalovirus promoter in a CRL4(DCAF1) E3 ligase independent manner

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ABSTRACT

Although the Vpr protein of human immunodeficiency virus type 1 (HIV-1) has been shown to act as a transcriptional activator of the HIV-1 LTR and certain host genes, the current study demonstrates that it can also function as a potent inhibitor of the cytomegalovirus (CMV) promoter. Previous studies have shown that the cell cycle arrest and apoptotic functions of Vpr required recruitment of the CRL4(DCAF1) E3 ligase, but this complex is shown not to be required for inhibition of the CMV promoter. We identified conserved sites (A30/V31) from diverse Vpr from HIV/SIV that were critical for blocking the CMV promoter activity. Interestingly, the Vpr mutant A30S/V31S protein also impaired the ability of Vpr to down-regulate transcription of the host *UNG2* gene. Our findings shed light on the dual functions of Vpr on the transcription of HIV-1, other viruses and host genes which may contribute to viral replication and disease progression *in vivo*.

1. Introduction

Human immunodeficiency viruses (HIV-1 and HIV-2) and simian immunodeficiency viruses (SIV) encode a conserved 14 KDa protein of 96 amino acids, the regulatory viral protein R (Vpr) [1–4]. Vpr and its homologous viral protein Vpx which is encoded by HIV-2 and SIV, are specifically packaged into virus particles through an interaction with the p6 region of the Gag precursor, indicating a role in the early steps of the virus life cycle [1,3,5,6]. More importantly, in SIV-infected monkeys, Vpr is required for viral dissemination and disease progression [7–9].

Vpr is known to be a multifunctional viral protein. The most investigated function of Vpr is its ability to prevent cell proliferation and arrest the cell cycle at the G2 phase [10]. Other HIV-1 accessory proteins, such as Vif and Vpu, have been shown to function by hijacking of the cellular E3 ubiquitin ligase to degrade the host restriction factors, APOBEC3G and BST-2/tetherin, respectively. By a similar mechanism Vpr has been shown to function by recruiting the host CRL4(DCAF1) E3 ligase to induce cell cycle arrest by degrading an as yet unidentified factor [11–15]. Recent studies have also indicated that Vpr interacts with and prematurely activates the host SLX4/MUS81/EME1 complex, which promotes cell cycle arrest and evasion of the innate immune response [16,17].

Vpr also triggers apoptosis of host cells, which contributes to the depletion of CD4+ T cells *in vivo* [18–20]. Although Vpr is not required for infection of activated T cells, it enhances viral replication in non-dividing monocytes [21,22]. In addition, Vpr also has several other functions such as dysregulation of mitochondria [23,24] and nuclear import of the viral preintergration complex [25].

Increasing evidence suggests that Vpr is a transcriptional regulator of the HIV-1 LTR [26] and an array of host genes, including *ung2* [27], *nlbp2* [28], *nhe1* [29] among others, which may enhance viral replication and disease progression *in vivo*. In the current study, we show that Vpr is a transcriptional suppressor of the

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promoter of cytomegalovirus (CMV), which is widely distributed in latent form among human populations. Further studies indicate that conserved sites in the first helix of HIV-1 Vpr are essential for Vpr-mediated inhibition of the transcription of both CMV and the host gene *ung2*. The potential implications for HIV-1 disease progression of Vpr's effect on viral and host gene transcription are discussed.

2. Materials and methods

2.1. Plasmid construction

The sequence of Vpr from HIV-1 NL4-3 stain cloned into the expression vector VR1012 with an HA tag at the N-terminus has been described previously [30].pVpr A30S/V31S and Q65R were made from pVpx-HFA by site-directed mutagenesis. Plasmids pSIVmus Vpr and pSIVdeb Vpr established in plasmid VR1012 has been described previously [31]. pCMV-GFP, with GFP sequences in the pcDNA3.1 vector, was purchased from Invitrogen (V795-20). The *ung2* promoter reporter vector p*ung2*-Luc S was a gift from Geir Slupphaug, Norwegian University of Science and Technology, Trondheim, Norway.

2.2. Antibodies and cell culture

The following antibodies were used anti-HA monoclonal antibody (MAb) (Covance, MMS-101R), anti-VPRBP (Shanghai Genomics,SG4220-28), anti-Green Fluorescent Protein (Tianjin Sungene Biotech, KM8009) and anti-actin monoclonal antibody (Sigma, A3853). HEK293T cells and Hela cells (AIDS Research Reagents Program) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin. All cultured cell lines were maintained at 37 °C in a humid atmosphere containing 5% CO2.

2.3. Transfection and immunoblotting

DNA transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions HEK293T cells were harvested at 36 h after transfection, washed twice with cold PBS, and lysed in lysis buffer (150 mM Tris, pH 7.5, with 150 mM NaCl, 1% Triton X-100, and complete protease inhibitor cocktail tablets [Roche]) at 4 °C for 30 min, then centrifuged at 10,000g for 30 min. Samples were then analyzed by SDS-PAGE and immunoblotting with the appropriate antibodies as previously described [30].

2.4. Luciferase assay

Plasmid sets comprised of the indicated plasmids were transfected into HEK293T cells; cells were harvested 36 h later Firefly luminescence was recorded with an Ultra Evolution plate reader (Tecan) with the use of the Dual-Luciferase Reporter Kit (Promega, E1910) according to the manufacturer's instructions.

3. Results and discussion

3.1. Vpr inhibits the transcription activity of the CMV promoter

CMV, a herpes virus, is highly prevalent in human populations as an asymptomatic infection. However, in immunocompromised individuals, such as those with AIDS, it is a major cause of morbidity and mortality [32]. Vpr has been shown to transactivate HIV-1 transcription and the affinity of its binding to the HIV-1 LTR has been correlated with specific virulence properties of the virus [33–36]. Given the adverse effects of CMV infection in the setting of immunodeficiency induced by HIV-1, we asked whether Vpr might also affect transcription of CMV. To address this issue, we constructed a CMV promoter reporter vector pCMV-Luc and transfected into HEK293T cells with or without pHA-Vpr. After 36 h, luciferase activity of the transfected cells were measured by recording firefly luminescence. Interestingly, our results showed that activity of the CMV promoter was significantly suppressed by Vpr expression (Fig. 1A and B). To further confirm this result, we examined the effect of Vpr on the CMV promoter by using the pCMV-GFP reporter vector. Vpr decreased both the detected fluorescence intensity (Fig. 1C) and the expression level of GFP (Fig. 1D). In addition, we also found that Vpr decreased GFP levels in pCMV-GFP transfected Hela cells (Fig. S1). Thus, using two independent expression systems and two different cell lines our studies revealed that HIV-1 Vpr inhibits the transcription of the CMV promoter.

3.2. CRL4(DCAF1) E3 ligase is not necessary for Vpr regulates CMV promoter activity

Previously, our lab and others have determined that DCAF1-binding is critical for Vpr function. The Vpr Q65R mutation, which abolishes Vpr and DCAF1 interaction, impaired Vpr-induced cell cycle arrest and apoptosis [11–13,30]. To examine whether Vpr recruitment of the CRL4(DCAF1) E3 ligase played a role in the Vpr-mediated inactivation of the CMV promoter, we compared in HEK293T cells the ability of Vpr wild type (WT) and Q65R mutant protein to inhibit CMV promoter transcription (Fig. 2A and B). Our data indicate that Vpr WT and Q65R are equally efficient in the suppression of CMV promoter activity, as measured either by fluorescence intensity (Fig. 2A, panel 2 and 3) or expression level of GFP (Fig. 2B, lane 2 and 3).

We next generated DCAF1 defective cells by using shRNA targeting *dcaf1*, which decreased the expression of endogenous DCAF1 protein in HEK293T cells by over 95% (Fig. 2D, lane 3 and 4). pCMV-GFP with or without pHA-Vpr was transfected into shDCAF1 cells and shControl cells, respectively. After 36 h, transfected cells were detected by immunofluorescence microscopy (Fig. 2C), and harvested for immunoblot assay (Fig. 2D). The results indicate that Vpr inhibited the activity of the CMV promoter independent of endogenous DCAF1 expression (Fig. 2C and D). Moreover, we found that the proteasome inhibitor MG132 did not alter the efficiency of Vpr inhibition of CMV transcription (Fig. S2), further confirming that regulation of the CMV promoter by Vpr is independent of the ability of Vpr to hijack the CRL4 E3 ubiquitin ligase.

3.3. A30/V31 are critical sites in Vpr-mediated transcriptional control of CMV and host ung2 promoter

To further characterize the mechanism by which Vpr specifically regulates CMV transcription, we performed mutational analysis of Vpr-mediated CMV transcriptional suppression. Recent studies from our laboratory found that a helix domain of Vpr was highly conserved in HIV-1 and SIV and that this domain was required for Vpr function [30]. Here we further identified two conserved sites (A30 and V31) in Vpr Helix 1 which were critical for its effect on the CMV promoter. In HEK293T cells, pCMV-Luc was transfected with plasmid encoding wild type Vpr, mutant A30S/V31S Vpr or the empty vector. After 36 h cells were harvested to measure the luciferase activity. Vpr A30S/V31S failed to inhibit the activity of the CMV promoter compared to wild type Vpr (Fig. 3A and B). In addition, Vpr A30S/V31S did not affect GFP expression from pCMV-GFP in HEK293T cells (Fig. 3C and D). Our results confirm that A30 and V31 in the first helix of Vpr (Fig. 3E), are critical for HIV-1 Vpr regulation of CMV transcription.

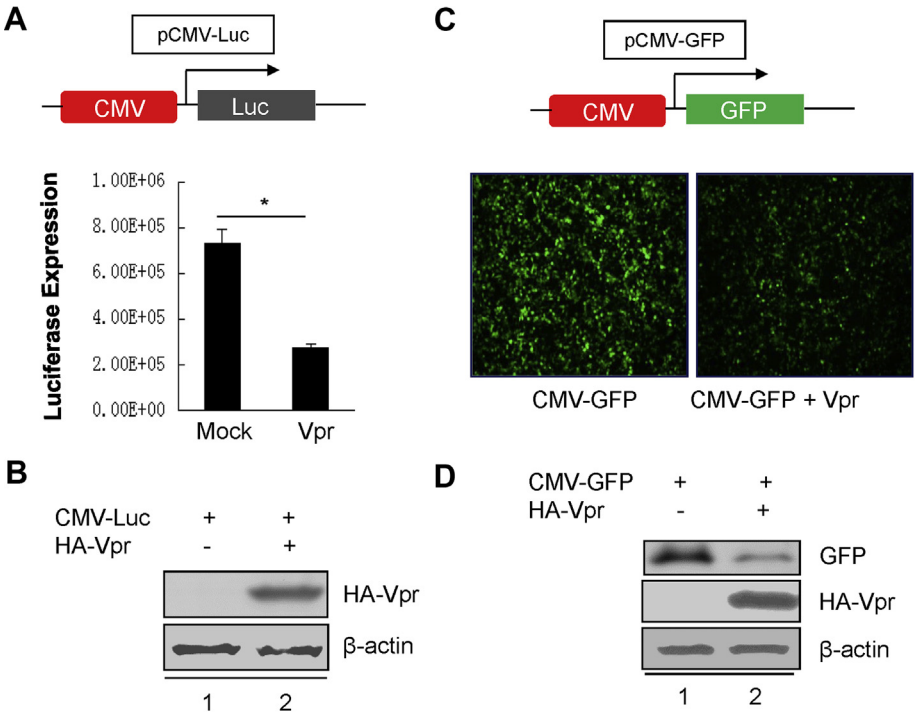

Fig. 1. HIV-1 Vpr inhibits the CMV promoter activity. (A) Luciferase assays confirmed the decreased transcriptional activity of the CMV promoter in the presence of Vpr expression. pCMV-Luc was transfected into HEK293T cells with or without pHA-Vpr, cells were harvested after 36 h later for luciferase assay (A) and immunoblot (B). Results are representative of three independent experiments. Each bar is the average of three replicates from the same experiment (error bars indicate standard deviations). (C) Vpr decreased the expression of green fluorescent protein in the pCMV-GFP reporter vector transfected cell. HEK293T cells were co-transfected with pCMV-GFP and pHA-Vpr WT or empty vector. Live cell imaging of the indicated cells at 36 h after transfection. (D) Cells from (C) were then prepared for an immunoblot assay with the indicated antibodies.

We next examined the impact of the A30/V31 mutation on Vpr regulation of host gene transcription. Vpr has previously been shown to down regulate UNG2 expression through protein degradation [11] and transcriptional inhibition [27]. In HEK293T cells, we studied the effect of Vpr WT and the A30S/V31S mutant on the *ung2* promoter. The results revealed that Vpr A30S/V31S impaired the negative transcriptional effect of Vpr on the expression of UNG2 (Fig. 4A and B). Thus the same conserved sites of Vpr are required for its transcriptional regulation of both a host gene and CMV.

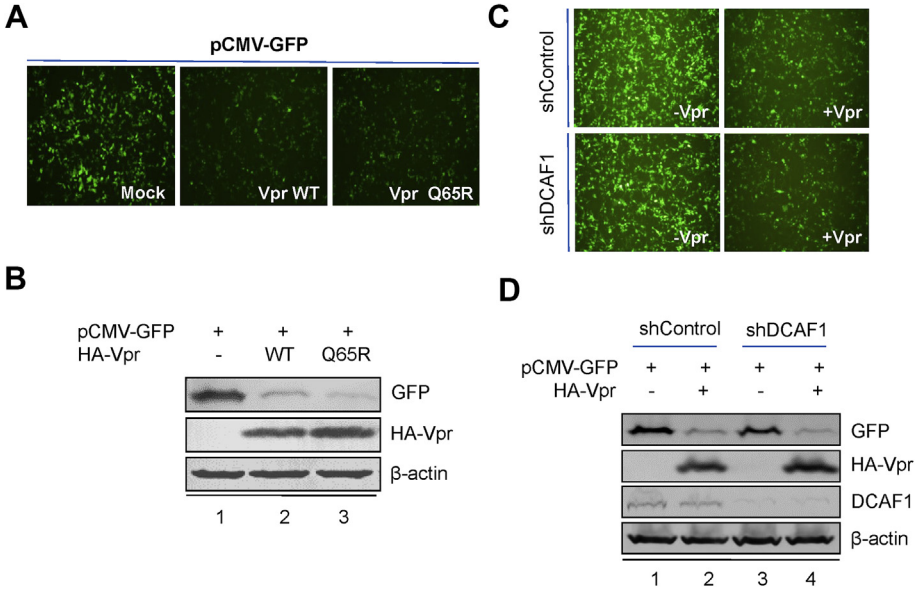

Fig. 2. Vpr-recruited CRL4(DCAF1) E3 ligase is unnecessary for Vpr-mediated downregulation of CMV transcription. (A) DCAF1-binding deficient Vpr mutant Q65R maintained the ability to modulate CMV promoter activity. HEK293T cells were co-transfected with pCMV-GFP and pHA-Vpr WT or Q65R or empty vector. Live cell imaging of indicated cells at 36 h after transfection. (B) Above cells were then prepared for immunoblot assay with the indicated antibodies. (C) Endogenous DCAF1 is dispensable for the suppression of CMV promoter by HIV-1 Vpr. DCAF1 knocked down cells were generated using DCAF1 targeted shRNA. pCMV-GFP and pHA-Vpr or empty vector were transfected into shDCAF1 cells and control cells respectively. Live cell imaging of the indicated cells at 36 h after transfection. (D) Above cells were then prepared for immunoblot assay with the indicated antibodies.

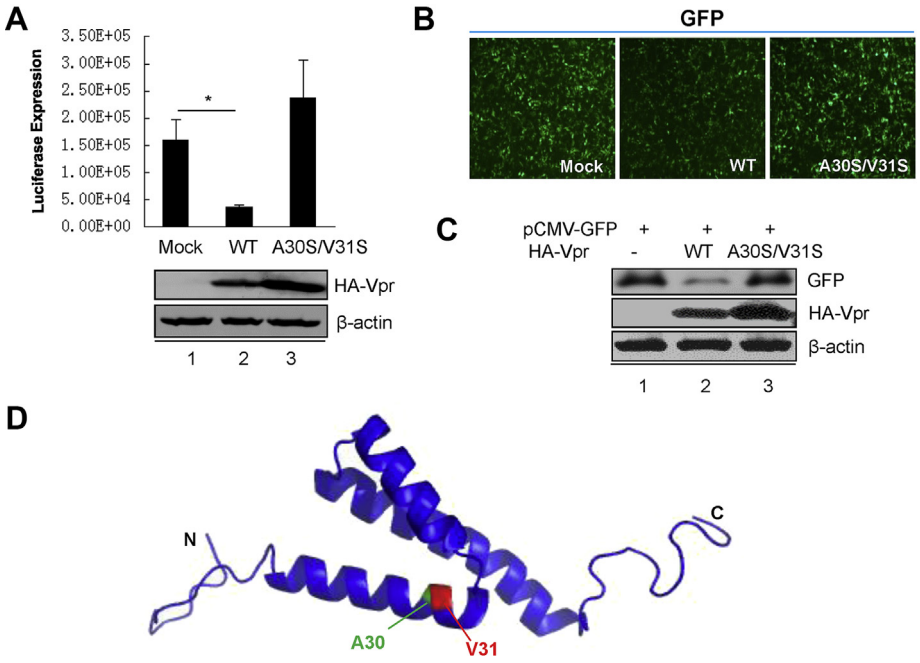

Fig. 3. A30/V31 are essential for the regulation of CMV promoter activity by HIV-1 Vpr. (A) Luciferase assays indicate that the A30S/V31S mutation impairs the ability of Vpr to down regulate CMV transcriptional activity. pCMV-Luc was transfected into HEK293T cells with pHA-Vpr WT or the A30S/V31S mutant, cells were harvested after 36 h later for luciferase assay (*Upper*) and immunoblot assay to detect Vpr expression (*Lower*). (B) and (C) Effects of Vpr A30S/V31S on the CMV promoter derived GFP expression. HEK293T cells were co-transfected with pCMV-GFP and Vpr WT or indicated mutant. Live cell imaging of indicated cells at 36 h after transfection. (C) Above cells were then prepared for immunoblot assay with the indicated antibodies. (D) Location of A30/V31 in the crystal structural model of HIV-1 Vpr (PDB: 1M8L).

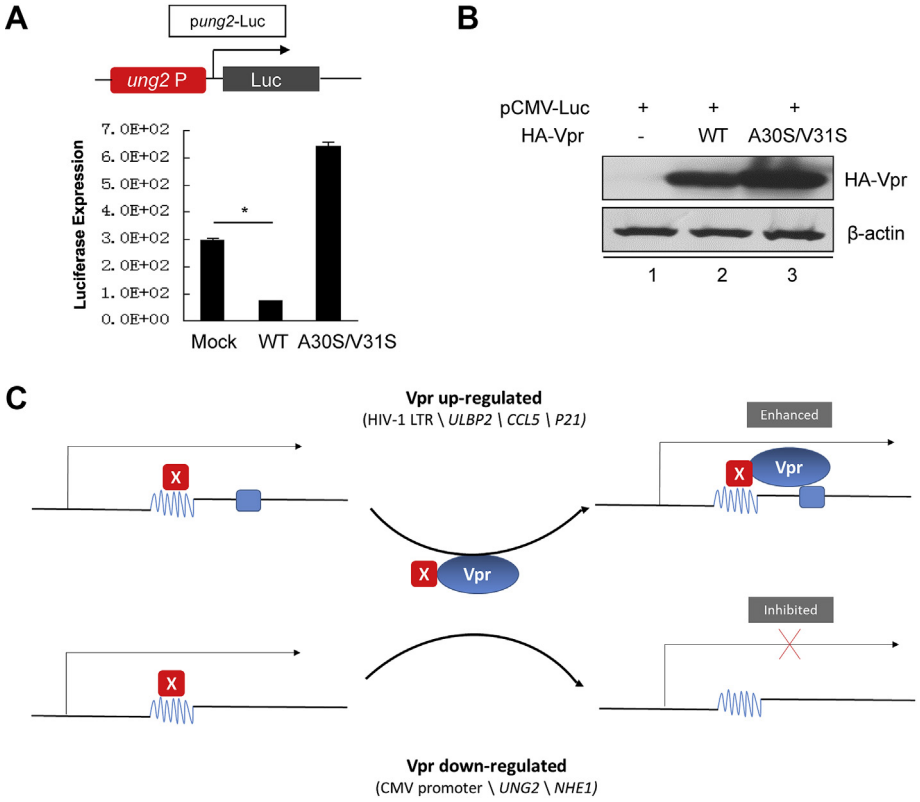

Fig. 4. A30/V31 are critical for Vpr down-regulation of host *UNG2* transcription. (A) Luciferase assays indicate that the A30S/V31S mutation impairs the ability of Vpr to down regulate *UNG2* transcriptional activity. p*UNG2*-Luc was transfected into HEK293T cells with pHA-Vpr WT or the A30S/V31S mutant; cells were harvested after 36 h later for luciferase assay (A) and immunoblot assay to detect Vpr expression (B). Results are representative of three independent experiments. Each bar is the average of three replicates from the same experiment (error bars indicate standard deviations). (C) Possible model of how Vpr hijacks a cellular transcription factor to Vpr-bound sequences for specific regulation of HIV-1, host genes and other viruses like CMV.

Interestingly, less evolutionarily evolved Vpr proteins from SIVmus and SIVdeb did not affect CMV transcription in our systems (Fig. S3). Consistent with this finding, the amino acid site V31 is not found in SIVmus Vpr (E31) or SIVdeb Vpr (M31).

Whether the evolution of Vpr to suppress CMV transcription is advantageous to HIV-1 or is due to another selection advantage attributable to this mutation is yet to be determined. It is known that CMV induces systemic immune activation during persistent infection [37]. The inhibition of CMV transcription *in vivo* at the early phase of HIV-1 infection could theoretically attenuate CMV-induced host anti-viral responses. Our lab and others have identified that Vpr and its homologous protein Vpx from diverse SIV strains have distinct evolutionary strategies to counteract the host restriction factor SAMHD1 [38,39]. Future studies to characterize the evolutionary path of the transcriptional regulatory functions of Vpr should provide further insights into how this virus has adapted to its current hosts.

The effects of Vpr on regulation of transcriptional activity are diverse, upregulating the transcription of the HIV-1 LTR [35], ULBP2 [40], CCL5 [41], and P21 [42], while downregulating that of NHE1 [29] and the CMV promoter. Previous studies demonstrated that Vpr regulates transcription through recruitment of host transcription factors (NF-kappaB, C/EBP et al.) [33]. Vpr has also been shown to bind chromatin DNA [43]. These findings suggest a model for how Vpr regulates transcription: Vpr hijacks critical host transcription factor(s) which it uploads onto Vpr binding DNA sequences (such as HIV-1 LTR) to promote the transcription process, while the activity of other promoters, which depend on the transcription factor(s) recruited by Vpr, are decreased (Fig. 4C).

Although Vpr is dispensable for HIV-1 infection of activated $CD4^+$ T cells, its ability to modulate transcription of both viral and host-cell proteins demonstrates its potential for influencing the course of HIV-1 infection [7–9]. Of particular note is the ability of soluble extracellular Vpr to enhance HIV-1 transcription in both cell lines and primary cells [44]. This ability to act *in trans* combined with the demonstrated effect of Vpr on transcription of non-HIV-1 viral as well as host genes indicates that the potential influence of Vpr may not be restricted to the cells directly infected by HIV-1. Further study of the activities of Vpr may provide important insights into the pathogenesis of HIV-1 infection and offer new opportunities for altering the disease course.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.060.

Transparency document

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