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Mutational analysis of HIV-2 Vpx shows that proline residue 109 in the poly-proline motif regulates degradation of SAMHD1

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ABSTRACT

In this study, we performed a mutational analysis to determine whether the mechanism by which HIV-2 Vpx confers the capacity for infectivity and viral replication in macrophages is solely dependent on its ability to degrade the host antiviral factor SAMHD1. Contrary to expectations, we demonstrated that P^{109} in the C-terminal poly-proline motif of HIV-2 Vpx has two unique roles: to facilitate the specific degradation of SAMHD1 in macrophages, and to facilitate multimerization of Vpx, therefore preventing SAMHD1 degradation in the presence of high levels of Vpx.

1. Introduction

Human immunodeficiency virus (HIV), which is the causative agent of AIDS, is classified into two types, HIV-1 and HIV-2 [1]. Each type has a unique set of accessory proteins: HIV-1 carries Vif, Vpr, Vpu and Nef, while HIV-2 carries Vif, Vpx, Vpr and Nef. After the studies of Vif [2–4], some accessory proteins have been shown to act as adaptors of the E3 ubiquitin ligase complex to antagonize host antiviral factors; thus, providing advantages in viral replication [5].

Vpx, an HIV-2-specific accessory protein, has been shown to be essential for virus replication in macrophages in vitro [6–11] and important in activated T lymphocytes [6,9,11,12]. Furthermore, Vpx is critical for SIV infection in monkeys in vivo [13–15]. Studies have indicated that significant amounts of Vpx packaged into virions [16–19] perform a function within the target cells. Vpx is critical for reverse transcription of the viral RNA genome in macrophages [10,20] and dendritic cells of macrophage lineage [21]. This process is induced by the formation of a Cul4-DDB1-DCAF1 E3 ligase complex and the proteasome-dependent degradation of a restriction factor [22–24], identified as SAMHD1 in 2011 [25,26]. Research indicated that SAMHD1 reduces deoxynucleotide triphosphate (dNTP) pools [27,28], which are components of viral genomic cDNA, and suppresses its reverse transcription in macrophages [29,30]. However, subsequent studies have indicated that the dNTPase activity of SAMHD1 is not absolutely required for its antiviral activity [31–33]. It was also reported that SAMHD1 has 3′ → 5′ exonuclease activity against ssDNAs and ssRNAs [34]. Recently, direct degradation of viral RNA mediated by the RNase activity of SAMHD1 was shown to be the dominant function of this protein, although the contribution of its dNTPase activity cannot be completely dismissed [35]. In addition to the function of Vpx in the degradation of SAMHD1, we hypothesized that Vpx possesses a SAMHD1-degradation independent function that enhances HIV-2 growth in T lymphocytes [36]. However, the latter function has not been demonstrated to influence viral growth in macrophages.

We have previously analyzed the replication of HIV-2 mutants carrying 19 point mutations in scattered regions of the *vpx* gene in macrophages [10]. Here, we investigated the ability of these mutants to degrade SAMHD1 in the human kidney cell line 293T, and compared their effects on viral infectivity in macrophages. A

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relatively good correlation was identified between these two characteristics among this panel of mutants. We next analyzed the properties of the mutants that were not related to this correlation, and unexpectedly found that the residue P^{109} within the C-terminal poly-proline motif (PPM) of Vpx plays a unique role in the regulation of SAMHD1 degradation.

2. Materials and methods

2.1. Plasmids

See Supplementary material.

2.2. Cell culture and transfection

The human kidney cell line 293T [40] was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), and the human monocytic cell line THP-1 [41] was cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS and 55 μM 2-mercaptoethanol. Monocyte-derived macrophages were prepared using GM-CSF as previously described [42]. Transfection of 293T cells with various plasmids was routinely performed by the calcium-phosphate co-precipitation method [43]. Exceptionally, transfection of full-length HIV-2 molecular clone pGL-AN or its mutant into 293T cells to observe cellular Vpx protein was performed using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA) to increase transfection efficiency. Transfected cells were incubated for 2 or 3 days post-transfection before use in experiments.

2.3. Immunoblot analysis

Cells were lysed in PBS-Laemmli sample buffer (1:1), and immunoblot analysis was performed as described previously [44]. As an antibody, anti-FLAG M2 (Sigma–Aldrich, St Louis, MO, USA) (1:1000), anti-β-actin clone AC-15 (Sigma–Aldrich) (1:1000), anti-myc-antibody (Life Technologies, Carlsbad, CA, USA) (1:1000), monoclonal anti-HA.11 clone 16B12 (Covance, Princeton, NJ, USA) (1:1000), anti-monomeric Kusabira–Green N-terminal fragment mAb (MBL, Nagoya, Japan) (1:1000), anti-monomeric Kusabira–Green C-terminal fragment mAb (MBL) (1:1000), HIV-2 Vpx monoclonal antibody 6D2.6 (NIH AIDS Research and References Reagent Program) [45] (1:1000), HIV-2 Rod polyclonal antibody (NIH AIDS Research and References Reagent Program) [46] (1:200), antiserum to SIV-p27 (NIBSC Centralized Facility for AIDS reagent) (1:1000), or anti-SAMHD1 antibody (Abcam, Cambridge, UK) (1:500) was used. Immunoreactivity was detected by chemiluminescence using ImmunoStar Zeta (for p27 detection), or ImmunoStar LD (for detection of the other proteins) (Wako Pure Chemical Industries, Osaka, Japan).

2.4. Bimolecular fluorescence complementation assay

The assay was performed using Kusabira–Green system [47]. For microscopic observation, all-in-one fluorescence microscope Biorevo BZ-9000 (Keyence, Osaka, Japan) was used (excitation, 470/40 nm; absorption, 535/50 nm).

2.5. Virion preparation and quantitative PCR analysis

Virions were prepared using Viro-Adembeads (Ademtech, Pessac, France) or ultracentrifugation using sucrose as described previously [48]. Quantitative PCR analysis was performed according to the same methods used in our previous report [10].

3. Results

3.1. Expression profiles of Vpx mutants

We previously performed mutational analysis using a series of mutants of the HIV-2 infectious clone pGL-AN [38] carrying 19 point mutations in scattered regions of Vpx (Fig. 1(a)) [10]. To examine the ability of the mutants to degrade SAMHD1, the mutations were introduced into pEF-Fvpx. This vector expresses high levels of Vpx carrying a FLAG-tag at its N-terminus [37], which has been reported to have no influence of the function of Vpx [49]. The pEF-Fvpx vector or its mutants were transfected into 293T cells, and expression from the mutant vectors was examined by western immunoblot analysis. As shown in Fig. 1(b), all the vectors expressed mutant Vpx, although the expression levels varied. Furthermore, two bands were observed for many of the mutant proteins. The upper band was considered to be attributed to phosphorylated Vpx; therefore, Phos-tag electrophoresis [50] followed by western immunoblot analysis of the mutants was performed. As shown in Fig. 1(c), the upper bands showed retarded migration in the electrophoresis. These proteins were treated then with lambda protein phosphatase, and analyzed by Phos-tag electrophoresis. In the presence of phosphatase, the upper bands were apparently lost, while the position of the lower bands did not change (Fig. 1(d)). These results clearly confirmed that the upper bands could be attributed to phosphorylated Vpx, while the lower bands were attributed to unphosphorylated Vpx.

3.2. The ability of Vpx mutants to degrade SAMHD1

To examine the ability of the mutants to degrade SAMHD1, 293T cells were co-transfected with 1.9 μg of pEF-Fvpx [37], and 1.4 μg of the myc-tagged human SAMHD1 expression vector (pcDNA hSAMHD1) or empty vector. Cells were lysed, and the steady-state expression levels of SAMHD1 were examined by western immunoblot analysis. Surprisingly, the expression levels of SAMHD1 with and without Vpx were almost identical. We speculated that the excessive amounts of Vpx protein expressed from 1.9 μg of pEF-Fvpx would prevent the degradation of SAMHD1. To test this hypothesis, the amount of pEF-Fvpx was gradually reduced from 1.9 μg to 0.0075 μg, and transfected into 293T cells with 1.4 μg of pcDNA hSAMHD1. The levels of SAMHD1 were then analyzed by western immunoblotting (Fig. 2(a)). SAMHD1 level was efficiently reduced in cells transfected with 0.03 μg or 0.06 μg pEF-Fvpx. These results showed that large amounts of Vpx prevent the degradation of SAMHD1; therefore, identification of the appropriate amounts of Vpx expression vector was important for our analysis. In subsequent experiments, we used 0.06 μg of pEF-Fvpx or its mutants. These vectors and pcDNA hSAMHD1 were co-transfected into 293T cells, and the steady-state expression levels were examined (Fig. 2(b)). The mutants P4L, N33S, E43G, C87A, H94A, P103A and P109A were found to degrade SAMHD1 with similar efficiency to that mediated by WT Vpx. Conversely, the other twelve mutants did not demonstrate the capacity to degrade SAMHD1 under these experimental conditions. To exclude the possibility that the amount of each mutant vector (0.06 μg) was insufficient to allow SAMHD1 degradation, we repeated these experiments using greater amounts (0.0075–1.9 μg) of mutant vectors. As shown in Fig. 2(c), the mutants P10L, E15G, E20G, W24L, W56L and Q76A did not degrade SAMHD1 under these experimental conditions. The other mutants reduced SAMHD1 levels with high or medium efficiency at specific concentrations of the vector.

These results, together with those of our previous mutational analysis [10] and the recently resolved crystal structure of the Vpx-SAMHD1-DCAF1 complex [51] are summarized in Fig. 2(d).

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