Zinc-binding site of human immunodeficiency virus 2 Vpx prevents instability and dysfunction of the protein

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Abstract

Human immunodeficiency virus 2 Vpx coordinates zinc through residues H39, H82, C87 and C89. We reported previously that H39, H82 and C87 mutants maintain Vpx activity to facilitate the degradation of SAMHD1. Herein, the expression of Vpx mutants in cells was examined in detail. We demonstrated that the zinc-binding site stabilizes the protein to keep its function in virus growth when low levels of Vpx are expressed. At higher levels of expression, Vpx aggregation could occur, and zinc binding would suppress such aggregation. Among the amino acids involved in zinc coordination, H39 plays the most critical role. In summary, zinc binding appears to mitigate flexibility of the three-helix fold of Vpx, thereby preventing dysfunction.

INTRODUCTION

Human immunodeficiency virus (HIV), which is the causative agent of AIDS, exists as two types, HIV-1 and HIV-2 [1]. HIVs have different sets of accessory proteins: HIV-1 has Vif, Vpr, Vpu and Nef; and HIV-2 has Vif, Vpr, Vpx and Nef. Vpr is found in HIV-1, whereas two homologous proteins, Vpx and Vpr, exist in HIV-2. Simian immunodeficiency viruses (SIVs), ancestors of HIVs, have evolved by frequent cross-species transmission [2]. Only Vpr is present in the oldest known SIV, and Vpr has evolved to Vpx and is found in SIVsmm (SIV carried by sooty mangabeys) together with Vpr, which was deleted at newer stages of evolution to become SIVcpz (SIV carried by chimpanzees) [3, 4]. During evolution, the functions of Vpr/Vpx have changed [5–8], although these proteins adopt a similar fold structure consisting of major three helices [9].

HIV-2 Vpx confers replication ability in macrophages [7] and resting T cells [10, 11], and enhances viral infectivity in activated T cells [7]. In macrophages/resting T cells, Vpx plays a functional role in the proteasome degradation of anti-viral host protein SAMHD1 [12, 13]. In mutational analysis, good correlation between ability to confer infectivity and that to degrade SAMHD1 was seen, and other functions of Vpx have not been found [14, 15]. Nonetheless, SAMHD1-independent functions of Vpx have been suggested in activated T cells [16, 17]; however, the details remain inconclusive.

In the degradation of SAMHD1, Vpx acts as an adaptor in the E3 ubiquitin ligase complex, which includes Cul4, DDB1 and DCAF1 [18]. Recently, the structure of Vpx complexed with the C-terminal domains of SAMHD1 and DCAF1 was solved [19]. In this structure, four amino acids of Vpx, H39, H82, C87 and C89, coordinate a zinc ion (Fig. 1a), which facilitates the folding of Vpx. We have reported that the expression level of the C87A Vpx mutant is extremely low in the context of the full genomic HIV-2. Nonetheless, the C87A mutant can facilitate degradation of SAMHD1 [14]. In this report, we examined the role of the zinc-binding site of HIV-2 Vpx in detail.

RESULTS

Expression level and function of zinc-binding site mutants of Vpx

The protein expression level of the FLAG-tagged C87A mutant of Vpx expressed from an EF-1α-promoter-driven expression vector was observed to be similar to that of WT, which differs from the low expression levels observed for the HIV-2 infectious clone pGL-AN [20]. This discrepancy was hypothesized to be due to the presence of the FLAG tag [14]. Thus, we first examined the expression level of H39L,
Fig. 1. Analysis of the expression level and function of zinc-binding site mutants of Vpx. (a) Position of the zinc-binding amino acids of Vpx of the GL-AN strain [20]. The gene originated from GH-123 [46], and an AvaI–NcoI fragment of pGH-123 (GenBank accession no. M30895) (nucleotides 6057–7943) was replaced by an AvaI–NcoI fragment of HIV-2ROD (GenBank accession no. M15390) (nucleotides 6059–7969). Vpx has 112 aa that fold into a three-helix conformer [9]. (b) Immunoblot analysis of proteins in 293T cells transfected with the expression vectors for Vpx (pEF-vpx) or its mutants (500, 100 or 50 ng). The total amount of vector used in each transfection was adjusted to 500 ng using the empty vector pEF1/Myc-HisA. (c) Immunoblot analysis of proteins in 293T cells transfected with pEF-vpx or its mutants (25 ng), with pEF1/Myc-HisA (left) or the expression vector for HIV-2 Gag (pEF-Fgag2) (right) (475 ng). (d) Immunoblot analysis of proteins in 293T cells transfected by the calcium-phosphate method with pGL-AN [20] (3.3 µg), its ΔVpx mutant pGL-St [20] (3.3 µg), or pGL-St (3.3 µg) and pEF-Fvpx (33 ng). (e) Immunoblot analysis of proteins in 293T cells transfected with pGL-AN or its mutants (500 ng). (f) Immunoblot of proteins in 293T cells co-transfected with pcDNA hSAMHD1 [14] (1.4 µg) and the expression vector for the FLAG-tagged Vpx C89A mutant (pEF-FxC89A) (0–1.9 µg). The total amount of vector used was adjusted to 3.3 µg using pEF1/Myc-HisA. (g) Luciferase activity of the lysate of phorbol-12-myristate-13-acetate-differentiated THP-1 cells infected with virus produced from 293T cells that were co-transfected by the calcium-phosphate method with a vpx- and env-deficient luciferase reporter clone [16] based on pGL-AN (5 µg), pCMV-G [40] (5 µg), and pEF1/Myc-HisA, pEF-vpx or its mutants (50 ng). Virus amounts were almost the same, and virus normalized by just 30 ng p27 was used in the infection. In (b–g), pEF1/Myc-HisA was used as the mock sample.
H82A, C87A and C89A (newly prepared) without the tag using EF-1α-promoter-driven expression vectors. To check the reactivity of the anti-Vpx antibody (HIV-2 Vpx mAb 6D2.6), four mutants of pEF-Fvpx (with FLAG tag) were transfected into 293T cells by the calcium-phosphate method, and Western immunoblot analysis using the anti-Vpx and anti-FLAG antibodies was performed. As shown in Fig. S1 (available in the online Supplementary Material), the anti-Vpx antibody reacts to all four mutant proteins and their phosphorylated form, which was examined in our previous study [14]. We then examined expression of the mutants in 293T cells transfected with pEF-vpx (without tag) mutants using the anti-Vpx antibody. To increase expression levels, the maximum amount (500 ng) of vector was transfected by the lipofection method (Fig. 1b). Surprisingly, all mutants containing C87A showed the same expression level as that of WT. This may be due to the high overexpression of Vpx. Thus, the amount of vector was reduced to 100, 50 (Fig. 1b) and 25 ng (Fig. 1c). At 50 ng, a difference between WT and mutants was observed, and at 25 ng the expression levels of the four mutants were clearly lower than that of WT. Since Gag has been shown to stabilize Vp [21], expression of Vpx and its mutants in the presence of HIV-2 Gag was examined. Fig. 1(c) shows that HIV-2 Gag increased the expression levels of Vpx and its mutants; however, a clear difference in the expression pattern between with and without Gag was not seen. It is noteworthy that bands of phosphorylated Vpx mutants were not seen in the absence of the tag, and the reason for this observation is under investigation.

The results presented suggest that the similar expression levels of the C87A mutant and WT presented previously [14] are not due to the use of the pEF-FxG87A vector. The expression from 25 ng pEF-Fvpx and its four mutants was then examined. As shown in Fig. S2, large differences in expression between WT and the mutants were observed, similarly to the expression of Vpx without the tag, thereby excluding the first hypothesis that the FLAG tag stabilizes the C87A mutant. Expression levels of Vpx using the EF-1α-promoter-driven expression vector and a full genomic infectious clone were then compared. As shown in Fig. 1(d), a 100-fold larger amount of pGL-AN expressed a smaller amount of Vpx than that of pEF-Fvpx, demonstrating that the amount of Vpx in a cell infected with HIV-2 is very small. Actually, when 293T was transfected with pGL-AN or its mutants, the pattern of expression (Fig. 1e) was similar to that expressed from a small (25 ng) amount of pEF-vpx (Fig. 1c). Notably, the expression level of H39L was the lowest among the four mutants in the experiments shown in Fig. 1(c, e). We showed previously that H39L, H82A and C89A can degrade SAMHD1 [14], and the newly prepared C89A mutant could also degrade SAMHD1, as shown in Fig. 1(f). As seen, 1.9 μg pEF-FxG89A did not work. We already reported that when there is a large amount of Vpx it loses its ability to degrade SAMHD1, similarly to the phenomenon of HIV-1 Vpr [22], and suggested that this is caused by multimerization of Vpx [14]. The range of vector concentrations to degrade SAMHD1 is smaller in WT, and wider in H82A, C87A and C89A. The details are under investigation. With regards to virus infectivity, a HIV-2 GL-AN clone carrying H39L mutated Vpx does not grow at all, and those having H82A and C87A mutations have severe growth defects, in macrophages [23]. C89A also had a defect similar to C87A, shown by a single-round replication assay using macrophage-like cells in Fig. 1(g). These results demonstrated the significance of the zinc-binding site of Vpx in maintaining sufficient amounts of protein in HIV-2 infection.

**Characteristics of low-level expression of Vpx zinc-binding-site mutants in cells**

We next investigated at which step the expression level of H39L, H82A, C87A and C89A becomes lower than that of WT Vpx by reducing the amount of the expression vector used. Initially, the transcription/translation step was examined in vitro transcription/translation, followed by Western immunoblot analysis using 500 ng pEF-vpx and its mutants. Fig. 2(a) shows that sufficient amounts of all mutant proteins were translated to a similar level as WT. There is the possibility that the transcription/translation result was due to the large amount of the vector used. Thus, the same in vitro experiment using much smaller amounts (25 ng) of vector was performed. To detect small amounts of Vpx, FLAG-tagged Vpx was used, because the anti-FLAG antibody is more sensitive than the anti-Vpx antibody. Immunoprecipitation after the in vitro transcription/translation experiment with anti-FLAG beads was performed. As shown in Fig. 2(b), the result was the same as that in Fig. 2(a). These results demonstrated that transcription and translation of H39L, H82A, C87A and C89A mutants were not dysfunctional.

The expression level of the protein shown in Fig. 1 was examined 2 days post-transfection. The expression of the protein at shorter incubation times (12 h) was investigated next. As shown in Fig. 2(c), almost the same expression level of H82A, C87A and C89A mutants as that of WT was seen. In contrast, the expression of the H39L mutant was barely observed, suggesting that H39L was degraded rapidly, and the other three mutants were degraded slowly following translation. We hypothesize that this degradation is caused by the proteasome. Thus, an experiment using a proteasome inhibitor was performed. To 293T cells transfected with 25 ng pEF-vpx or its mutants and pEF-Fag2 (expression vector for HIV-2 Gag), 5 μM clasto-lactacystin-β-lactone (its active form in a cell is lactacystin) was added, and after incubation for 17 h, the cells were harvested. Western immunoblotting analysis showed no clear recovery of the mutants (Fig. 2d). HIV-1 Vif is degraded by the proteasome [24]. Thus, the 293T cells were transfected with the expression vector for Vif and the cells were treated with clasto-lactacystin-β-lactone using the same conditions as in Fig. 2(d). As shown in Fig. S3, Vif increased, indicating that the Vpx mutants in Fig. 2(d) were degraded by a process different from proteasome degradation. Furthermore,
recovery of the mutant expression using the lysosome inhibitor bafilomycin A1, cysteine protease inhibitor E-64, serine protease inhibitor AEBSF or aspartic acid protease inhibitor pepstatin A was also not observed (data not shown).

The effect of mutation within the zinc-binding residues, from H to C, and from C to H, was examined next. Reactivity of the anti-Vpx antibody to these mutants was confirmed (Fig. S4). Then, expression levels of Vpx mutants in 293T cells transfected with 25 ng pEF-vpx or its mutants was examined. As shown in Fig. 2(e), expression levels of these mutants were lower than that of WT, indicating that the native sequence is important in defining expression levels of Vpx.

As the zinc-binding residues of Vpx influence protein expression levels, as shown above, expression of Vpx in cells is considered to be regulated by oxidative stress, since the SH group of cysteine is easily oxidized to disrupt the zinc-binding site. 293T cells transfected with 25 ng pEF-vpx were

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**Fig. 2.** Analysis of low-level expression of Vpx zinc-binding-site-mutants in cells. (a) Immunoblot analysis of proteins obtained after in vitro transcription/translation using expression vectors for Vpx (pEF-vpx) and Vpx mutants (500 ng). (b) Immunoblot analysis of proteins obtained after in vitro transcription/translation using expression vectors for FLAG-tagged Vpx (pEF-Fvpx) and its mutants (25 ng), followed by immunoprecipitation using anti-FLAG affinity gel. Detection of Vpx was achieved using anti-FLAG antibody. (c) Immunoblot analysis of proteins in 293T cells transfected with pEF-vpx or its mutants (25 ng) and the empty vector pEF-1/Myc-HisA (475 ng). The cells were harvested 12 h post-transfection. (d) Immunoblot analysis of proteins in 293T cells transfected with pEF-vpx or its mutants (25 ng) and pEF-1/Myc-HisA (475 ng) in the absence or presence of clasto-lactacystin-β-lactone (5 µM). This compound was added 3 h post-transfection and after 17 h incubation the cells were harvested. (e) Immunoblot analysis of proteins in 293T cells transfected with pEF-vpx or its mutant (25 ng) and pEF1/Myc-HisA (475 ng). (f) Immunoblot analysis of proteins in 293T cells transfected with pEF-vpx (25 ng) and pEF-1/Myc-HisA (475 ng) in the absence or presence of hydrogen peroxide (100–800 µM). This chemical was added to the cells 24 h post-transfection and after 24 h incubation the cells were harvested. (g) Luciferase activity of 293T cellular lysate co-transfected with firefly luciferase reporter vector having an AP-1 response element, pGL4.44[luc2P/AP1 RE/Hygro] vector (300 ng), and Renilla luciferase reporter vector having a β-actin promoter, pRL-Luc (200 ng), in the absence or presence of hydrogen peroxide (100–800 µM). Hydrogen peroxide was added to the cells 24 h post-transfection and after 24 h incubation the cells were harvested. Firefly luciferase activity was normalized to Renilla luciferase activity in order to calculate the relative luciferase activity. In (a–f), pEF1/Myc-HisA was used as the mock sample.
treated with the oxidizing reagent hydrogen peroxide and the expression level of Vpx was examined. As shown in Fig. 2(f), oxidative stress decreased the protein expression level, supporting the hypothesis that oxidative stress regulates Vpx expression. In contrast, HIV-2 Gag protein expressed from the vector having the same promoter did not decrease under the same conditions (Fig. S5), excluding a possibility that this stress does not affect transcription of the vpx gene. Notably, generation of oxidative stress by 400 and 800 μM hydrogen peroxide was proved by activation of transcription factor AP-1 [25–27], as shown in Fig. 2(g).

**Characteristics of high-level expression of Vpx zinc-binding-site mutants in cells**

We next examined why highly overexpressed H39L, H82A, C87A and C89A mutants had the same expression level as WT Vpx, as shown in Fig. 1. A certain amount of another HIV accessory protein, Vif, has been reported to exist in an insoluble fraction in cells and this insoluble fraction is stable [24]. 293T cells transfected with 25 ng pEF-vpx or its mutants were fractionated into cytoplasm, soluble nucleus and insoluble nucleus. As shown in Fig. 3(a), fractionation was confirmed by detection of control proteins HSP70 (cytoplasm), PARP-1 (soluble nucleus) and histone H3 (insoluble nucleus). WT Vpx and its mutants were observed in the cytoplasm fraction (Fig. 3a). Cellular fractionation of the protein expressed using 500 ng vector was next examined. As shown in Fig. 3(b), WT protein existed primarily in the cytoplasmic and soluble nuclear fractions, and a smaller amount was found in the insoluble nuclear fraction. In contrast, most of the H39L, H82A and C89A mutants were found in the insoluble nucleus fraction, whereas C87A was found mainly in the cytoplasmic fraction. In the nucleus, C87A protein also existed predominantly in the insoluble fraction. These results showed that the mutants had the tendency to be in an insoluble form. Furthermore, the status of the mutants in a cell was observed by confocal microscopy using HeLa cells, because 293T cells are difficult to use in this experiment owing to their weak adhering properties to slides. The results of the microscopy showed that cells expressing the H39L mutant showed clear protein aggregates, which were not observed for cells expressing the WT protein (Fig. 3c).

**DISCUSSION**

Zinc is coordinated by various proteins – e.g. HIV proteins NC [28, 29], integrase [30, 31] and Vif [32, 33] are zinc-binding proteins – and zinc coordination by these proteins has a functional role. Vpx was also recently reported to coordinate zinc [19]. In this report and a previous study [14], the zinc-binding site of Vpx was shown not to be required for degradation of SAMHD1. However, zinc coordination by Vpx was important for regulating protein expression to keep its function and a low level of Vpx expression was observed for full genomic HIV-2. The mechanism that controls the low-level expression of the non-zinc-binding mutants is unclear, but it was suggested that the mutants are unstable after translation. Notably, among the four amino acids mutated, H39 played the most critical role in protein stability. Conversely, when a high level of Vpx was expressed, the zinc-binding site of Vpx was important for reducing the formation of the insoluble form, which is probably due to aggregation.

Vpx is a small protein that adopts a three-helix fold and binds zinc, with residues H39, H82, C87 and C89 coordinating the ion [19]. Among these four amino acids, H39 is the most C-terminal amino acid of helix 1, whereas H82, C87 and C89 are located on helix 3 (Fig. 1a). Zinc stabilizes the structure between the loop positioned between helices 1 and 2 and helix 3, thereby mitigating structural flexibility of Vpx without zinc. A positive correlation of protein structural disorder and instability [34, 35] or aggregation [36, 37] has been reported. Herein, we have shown that the zinc-binding site confers stability, and a suggested function of zinc binding is to decrease Vpx aggregation. Since H39 is located at the C-terminal end of helix 1, this amino acid probably suppresses greater intrinsic flexibility in WT Vpx when compared with the other amino acids. This flexibility would not be fully quenched following complete zinc coordination. Importantly, some level of expression of WT Vpx has the propensity to be insoluble (Fig. 3b).

All Vpr/Vpx proteins of SIVs/HIVs may have elements of intrinsic structural disorder, and this probably facilitates the functional evolution of these proteins. However, this structural flexibility and disorder indicates protein instability, which may be of functional significance when the expression level of the protein is low, whereas at high levels of expression such weak structural integrity is likely to promote multimerization and aggregation, which lead to loss of activity. Thus, Vpx/Vpr protein expression levels would likely be regulated by a mechanism to avoid such an aggregation processes. This study suggests that one mechanism of Vpx to avoid aggregation is to coordinate zinc and thus adopt a more-stable folded state. Future efforts will aim to reveal further aspects of the relationship between evolution and the structural character of Vpr/Vpx proteins.

**METHODS**

**Plasmids**

The C89A mutation in the vpx sequence was introduced into the XbaI–NsiI fragment of pGL-AN [20] inserted into pUC19 by QuikChange site-directed mutagenesis (Agilent). The fragment was cloned into pGL-AN to yield the pGL-xC89A construct. The other mutants of pGL-AN, pGL-xH39L, pGL-xH82A and pGL-xC87A, were constructed previously [23]. The ΔVpx mutant of pGL-AN, pGL-St, was described previously [20], and the vpx- and env-deficient luciferase reporter clone [16] based on pGL-AN was given to us by Dr Akio Adachi, Tokushima University (Tokushima, Japan). A plasmid encoding an N-terminal FLAG-tagged Vpx C89A mutant, pEF-FxC89A, was constructed by replacement of pEF-Fvpx [38] with the mutant vpx fragment. The fragment was amplified by PCR using
pGL-xC89A, and included EcoRV and XbaI sites at the 5′ and 3′ termini, respectively. H39C, H82C, C87H or C89H mutations in the vpx sequence were introduced into the EcoRV–XbaI fragment of pEF-Fvpx inserted into pBlue-script SK(+) (Agilent) by QuikChange site-directed mutagenesis, and the fragments were cloned into pEF-Fvpx to yield pEF-FxH39C, pEF-FxH82C, pEF-FxC87H and pEF-FxC89H, respectively. The other mutants of pEF-Fvpx, pEF-FxH39L, pEF-FxH82A and pEF-FxC87A were constructed previously [14]. Plasmids encoding Vpx without

Fig. 3. Analysis of high-level expression of Vpx zinc-binding-site mutants in cells. (a) Immunoblot analysis of proteins in 293T cells transfected with the expression vectors for Vpx (pEF-vpx) or its mutants (25 ng) and the empty vector pEF1/Myc-HisA (475 ng), followed by cellular fractionation. Cy, Cytoplasmic fraction; IN, insoluble nucleus fraction; SN, soluble nucleus fraction. (b) The same experiment as that in (a) except using 500 ng pEF-vpx and its mutants. (c) Confocal microscopy analysis of the Vpx protein in HeLa cells transfected with pEF-vpx or its H39L mutant (500 ng). In all experiments shown in this figure, pEF1/Myc-HisA was used as the mock sample.
the tag, pEF-vpx, and its mutants, pEF-xH39L, pEF-xH82A, pEF-xC87A, pEF-xC89A, pEF-xH39C, pEF-xH82C, pEF-xC87H and pEF-xC89H, were constructed by insertion of the vpx fragment or its mutants into pEF1/Myc-HisA (Thermo Fisher Scientific). The fragment was amplified by PCR using pGL-AN and its mutants (H39L, H82A, C87A, C89A). The mutant vpx fragments were inserted into pBlue-script-SK(+) (H39C, H82C, C87H, C89H), which included EcoRI and XbaI sites at the 5′ and 3′ termini, respectively. To construct a plasmid encoding N-terminal FLAG-tagged HIV-2 Gag, pEF-Fgag2, a gag fragment encoding a region from MA to p6 was amplified by PCR using pGL-AN, which incorporated EcoRV and XbaI sites at the 5′ and 3′ termini, respectively. Since the gag fragment contained two original EcoRV sites, treatment of the fragment amplified by PCR with EcoRV and XbaI enzymes generated three fragments, EcoRV–EcoRV (fragment 1, 5′ terminus), EcoRV–EcoRV (fragment 2, middle) and EcoRV–XbaI (fragment 3, 3′ terminus). Finally, fragment 3 was replaced with the vpx sequence of pEF-Fvpx, and then fragment 2 was inserted into the vector, followed by silent modification of the EcoRV site at the 3′ site of fragment 2 by QuikChange site-directed mutagenesis. Fragment 1 was inserted into the vector to yield pEF-Fgag2. To express human SAMHD1 and HIV-1 Vif, pcDNA hSAMHD1 [14] and pNL-ASCF [39] were used. To generate vesicular stomatitis virus G protein (VSV-G) pseudotyped virus, pCMV-G [40] was used. For the reporter assay, pGL4.44[luc2P/API1/RE/Hygro] vector (firefly luciferase reporter vector having an AP-1 response element) (Promega) and pRL-Luc (Renilla luciferase reporter vector having a β-actin promoter) [41] were used. In the cell constructs, the sequence was verified by DNA sequencing, following QuikChange site-directed mutagenesis or PCR amplification.

**Cell culture, differentiation, transfection and reagents**

The human kidney cell line 293T [42] was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FBS. The human monocytic cell line THP-1 [43] was cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS and 55 μM 2-mercaptoethanol. THP-1 cells were differentiated by stimulation with phorbol-12-myristate-13-acetate (Sigma-Aldrich) at 300 nM and incubation for 3 days. The human cervical cancer cell line HeLa [44] was maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% FBS. Transfection of 293T and HeLa cells with various plasmids was performed using Lipofectamine 3000 (Life Technologies), unless otherwise stated. Clasto-lactacystin-β-lactone (Sigma-Aldrich) and hydrogen peroxide (Wako Pure Chemical Industries) were added to cell cultures. Transfected cells were incubated for 2 days post-transfection before use in experiments, unless stated otherwise.

**ELISA, infection and luciferase assay**

Virus amounts were determined by SIV p27 antigen ELISA kit (ZeptoMetrix). Cells were infected with VSV-G pseudo virus in the presence of DEAE-dextran (5 µg ml⁻¹) (PK Chemicals). The luciferase assay was performed using the Luciferase assay system and Renilla Luciferase assay system (Promega).

**In vitro transcription/translation and immunoprecipitation**

For in vitro transcription/translation, the TNT T7 quick coupled translation system (Promega) using a rabbit reticulocyte lysate was used. To conduct immunoprecipitations, the reaction mixture was diluted (20×) in TNE buffer (10 mM Tris-HCl, pH 7.8, 0.15 M NaCl, 1 mM EDTA, 1% NP40, 10 µg aprotinin ml⁻¹) and mouse anti-FLAG M2 affinity gel (Sigma-Aldrich) was added. The suspension was incubated at 4°C for 20 h. The gel was washed and analysed by Western blotting. Using the supernatant following the incubation of the in vitro reaction mixture and anti-FLAG beads, further immunoprecipitation was attempted to check that the supernatant did not contain Vpx protein.

**Cellular fractionation experiment and immunoblot analysis**

Fractionation of cells was performed using a LysoPure nuclear and cytoplasmic extraction kit (Wako Pure Chemical Industries). Immunoblot analysis was conducted using cells lysed in PBS/Laemmli sample buffer (1:1), as described previously [24]. The antibodies anti-FLAG M2 (Sigma-Aldrich) (1:1000), HIV-2 Vpx mAb 6D2.6 (NIH AIDS Research and Reference Reagent Program) [45] (1:1000), anti-β-actin clone AC-15 (Sigma-Aldrich) (1:1000), antiserum to SIV-p27 (NIBSC Centralized Facility for AIDS Reagents) (1:1000), anti-Myc-antibody (Life Technologies), HSP70/HSC70 (W27) (Santa Cruz Biotechnology) (1:1000), anti-PARP-1 (Ab-2) mouse mAb (C-2) (Chemicon) (1:1000) or anti-histone H3 mAb (Medical and Biological Laboratories, Nagoya, Japan) (1:500) were used. Immunoreactivity was detected by chemiluminescence using ImmunoStar Zeta (for detection of p27 and histone H3) or ImmunoStar LD (for detection of the other proteins) (Wako Pure Chemical Industries). When using the anti-PARP-1 antibody, a band was also observed in the cytoplasm fraction; however, the band position was lower than that in the soluble nucleus fraction.

**Confocal microscopy analysis**

Cells were grown on chamber slides, fixed in a 4% PBS solution of paraformaldehyde (Wako Pure Chemical Industries) by incubation for 15 min at room temperature, and washed with PBS three times. The cells were then incubated with PBS containing 5% normal goat serum (Wako Pure Chemical Industries) and 0.3% Triton X-100 (Nacalai Tesque) for 1 h at room temperature, and reacted with the HIV-2 Vpx mAb 6D2.6 (NIH AIDS Research and Reference Reagent Program) [45] (1:400) in PBS containing 1% BSA and 0.3% Triton X-100 for 2 h at room temperature. After washing with PBS three times, the cells were reacted with goat anti-mouse IgG (H+L) secondary antibody-Alexa Fluor 488 (Thermo Fisher Scientific). The cells were washed.
with PBS three times and mounted in Fluoromount-G (Agilent). Images were acquired using a TCS SP5 confocal laser-scanning microscope (Leica).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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