HHS Public Access

Author manuscript

*Methods Mol Biol*. Author manuscript; available in PMC 2017 June 22.

Published in final edited form as:

*Methods Mol Biol*. 2014 ; 1087: 125–134. doi:10.1007/978-1-62703-670-2_11.

# HIV-1 Accessory Proteins: VpR

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## Abstract

HIV-1 viral protein R (VpR) is a multifunctional protein that plays specific roles at multiple stages of the HIV-1 viral life cycle and affects anti-HIV functions of the immune cells. VpR is required for efficient viral replication in nondividing cells such as macrophages, and it promotes, to some extent, viral replication in the proliferating target CD4+ T cells. A number of specific activities that may contribute to these effects of VpR have been proposed. In this chapter, we describe two best characterized activities of VpR, nuclear import of the HIV-1 preintegration complex (PIC) and induction of cell cycle G2 arrest, focusing on the methods used for their demonstration.

### Keywords

HIV-1; Vpr; Cell cycle G2 arrest; Nuclear transport; Fission yeast

## 1 Introduction

Human immunodeficiency virus type 1 (HIV-1) VpR is a virion-associated accessory protein with an average length of 96 amino acids and a calculated molecular weight of 12.7 kDa. VpR is involved in protein–protein interactions with a number of cellular proteins, and these interactions underlie multiple effects that VpR has on viral replication and cell physiology, including modulation of fidelity of viral reverse transcription and nuclear import of the HIV-1 preintegration complex (PIC), transactivation of the HIV-1 LTR promoter, induction of cell cycle G2 arrest and apoptosis. We focus here on two best characterized activities of VpR, nuclear import of PIC and induction of G2 arrest.

### 1.1 Role of VpR in Nuclear Transport of HIV-1 Preintegration Complex

One of the unique VpR activities is its ability of cytoplasmic-nuclear shuttling [1], which is believed to contribute to nuclear transport of the viral PIC [1–3]. To infect a host cell, HIV-1 needs to transport its genomic DNA in the context of the viral PIC from the cytoplasm into the nucleus of a target cell. VpR is believed to be among the main regulators of HIV-1 nuclear import by directly associating with PIC and connecting it to cellular nuclear import machinery [1, 4]. In normal cells, nuclear transport of proteins involves a 2-step process, which includes an energy-independent docking of the cargo protein to the nuclear envelope and the subsequent energy-dependent translocation and release of the cargo protein into the nucleus. The imported protein is generally required to carry a nuclear localization sequence (NLS) domain that consists of a short region of basic amino acids (lysines and arginines) or two such regions spaced about ten amino acids apart [5, 6]. Typically, the importin α tags

the NLS-containing protein, and serves as a bridge between the cargo and the receptor importin β through the importin β-binding domain (IBB) on importin α. The transport process involves cytoplasmic-nuclear shuttling of the ternary protein complex and subsequent release of the cargo protein in the nucleus [7, 8]. The exact activity of VpR in PIC nuclear import process is a subject of intense debates, but three main hypotheses have been proposed. The first model hypothesizes that VpR targets the HIV-1 PIC to the nucleus via a distinct, importin-independent pathway [9, 10]; the second suggests that VpR modifies cellular importin-dependent import machinery including both importin α and importin β [3]; and the third proposes that VpR activity allows HIV-1 PIC to use importin α pathway without involvement of importin β [11]. The methods used to demonstrate these VpR activities include analysis of nuclear import of fluorescently tagged VpR and PIC, inactivation of various components of nuclear import machinery by antibodies or RNAi, and reconstruction of nuclear import using digitonin-permeabilized cells. The latter technique provides an opportunity to directly demonstrate the role of particular components of the nuclear import system and will be described below.

### 1.2 Role of VpR in the Induction of Cell Cycle G2 Arrest

Another unique activity of HIV-1 VpR is its ability to inhibit host cell proliferation by blocking infected cells in the G2/M phase of the cell cycle, which is commonly known as the G2 arrest [12–14]. The cell cycle G2 arrest induced by VpR is thought to suppress human immune function by preventing T-cell clonal expansion [15] and to provide an optimized cellular environment for maximal levels of viral replication [16]. However, contribution of VpR to viral replication in proliferating T cells is relatively small [16, 17], and no direct evidence has been provided to demonstrate the role of VpR in preventing T-cell clonal expansion. Thus, the virological role of VpR-induced G2 arrest remains unproved.

The mechanisms that trigger VpR-induced G2 arrest have been controversial. Since there are two well-conserved cell cycle DNA damage or replication checkpoint surveillance systems in eukaryotic cells engagement of which could lead to cell cycle G2 arrest, debates have been focused on which one of the two checkpoints is responsible for VpR-induced G2 arrest. Another possibility is that VpR itself could also actively cause cell cycle G2 arrest of host cells. The main confusion comes from the fact that available data support all of these possibilities. For example, the eukaryotic cell cycle DNA damage or replication checkpoint controls, as well as VpR, all induce G2 arrest through inhibitory phosphorylation of CDK1 that is regulated by CDC25 or WEE1. Thus, it is logical to think that VpR might induce G2 arrest through one of these two checkpoint pathways (for detailed reviews, *see* ref. 18–20). Consistent with this notion, VpR induces DNA double-strand breaks (DSBs), which support the idea that VpR induces G2 arrest through DNA damage checkpoint [21]. However, expression of *vpr* does not change radiosensitivity of the checkpoint defective mutants [22] or increase gene mutation frequency [23], arguing against the possibility that VpR actually causes DNA damage. Similarly, another report showed that VpR does not induce DNA DSBs [24]. Moreover, downregulation of H2AX, a hallmark of DSBs, had little or no effect on VpR-induced G2 arrest, suggesting that this process is a late event and the G2 induction is most likely independent of DNA damage checkpoint [25]. On the other hand, the ATR

kinase instead of the ATM kinase was found to play a major role in VpR-induced G2 arrest through activation of Chk1 via S345 phosphorylation [25–28]. These studies suggested that VpR-induced G2 arrest may in fact resemble more the activation of DNA replication checkpoint than the DNA damage checkpoint control. Further studies have shown numerous similarities between the ATR-dependent pathway activated by VpR and by HU/UV. These similarities include the requirement for Rad17 and Hus1, the induction of phosphorylation on Chk1 and the formation of nuclear foci by RPA, 53BP1, BRCA1, and γH2AX [27–29], all of which indicate activation of DNA replication checkpoint control. However, these findings remain inconclusive because activation of DNA replication checkpoint generally leads to S phase arrest but not G2 arrest.

One of possible contributing factors for the reported controversies in examining this molecular event is that most of those studies on VpR-induced G2 arrest measured the VpR effect 48–72 h after introduction of VpR in an asynchronized cell population. With this single late time point, it is not possible to distinguish which events precede and therefore might cause the G2 arrest, and which events happen after the initiation of G2 arrest and therefore are the result but not the cause of the G2 arrest. To facilitate this study, measurement of the initiating event(s) for VpR-induced G2 arrest would benefit from a system that uses synchronized cells and minimizes the time between initiation of VpR expression and measurement of the G2 arrest. For this reason, we have adapted an approach that allows us to monitor the cellular signaling for VpR-induced G2 arrest within 11 h of a single cell cycle [30]. The specific experimental procedure is described below.

Another way to study the effect of HIV-1 VpR on the induction of cell cycle G2 arrest is to use fission yeast (*Schizosaccharomyces pombe*) as a model system. Abundant evidence has been accumulated to indicate that induction of cell cycle G2 arrest by VpR is a highly conserved activity between human and fission yeast [20, 31–33]. The advantage of using a fission yeast model system is that it can be grown and maintained easily in the laboratory. In addition, it also has an inducible promoter that allows specific *vpr* gene expression under controlled conditions (see specific procedures below).

## 2 Materials

Prepare all solutions using deionized water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

### 2.1 Role of VpR in Nuclear Transport of HIV-1 Preintegration Complexes

1. Hypotonic buffer—5 mM HEPES, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotinin, pepstatin, leupeptin. Keep at 4 °C.
2. Transport buffer—2 mM MgOAc, 20 mM HEPES pH 7.3, 110 mM KOAc, 5 mM NaOAc, 1 mM EGTA, and 2 mM DTT. Keep at 4 °C.
3. Mixed medium—199 Eagle medium, 2 % $NaHCO_3$, 20 mM L-glutamine, 13 nonessential amino acids, 10 mM sodium pyruvate. Keep at 4 °C.

4. Digitonin (Fluka)—prepare a 20 mM stock solution in DMSO.
5. Energy regenerating system—5 mM creatine phosphate, 20 U/ml creatine phosphokinase, 1 mM ATP, 0.1 GTP, 1 μg/ml each aprotinin, leupeptin, and pepstatin.

### 2.2 Role of VpR in the Induction of Cell Cycle G2 Arrest

1. Thymidine—prepare 200 mM stock solution in water, sterile filtered. Keep at −20 °C.
2. RNase A—prepare 10 mg/ml stock solution in water. Keep at −20 °C.
3. Propidium iodine (PI)—prepare 1 mg/ml stock solution in water. Keep at 4 °C.

## 3 Methods

### 3.1 Analysis of the Role of VpR in HIV-1 PIC Nuclear Import in Digitonin-Permeabilized Cells

This experimental approach exploits the ability of a nonionic detergent digitonin (a glycoside obtained from *Digitalis purpurea*) to permeabilize cell plasma membrane without significantly affecting properties of the nuclear membrane. Cytosolic components can be washed out of permeabilized cells, followed by addition of nuclear import factors and energy source in any combination, allowing reconstitution of the nuclear import. This system has been used to analyze nuclear import of fluorescently tagged HIV-1 proteins and PIC, but import can be also monitored by PCR [3].

1. First, prepare the cytoplasmic lysates of uninfected cells that will serve as source of nuclear import factors [34]. Harvest HeLa cells, wash twice with ice-cold PBS, resuspend in ice-cold hypotonic buffer and let the cells swell on ice for 10 min (total volume ~40 ml). Lyse the cells on ice by adding digitonin to the point where ~90–95 % of the cells are permeable to Trypan Blue (*see* Note 1). Remove permeabilized cells and debris by centrifugation at 1,500 × *g* for 15 min at 4 °C, collect the supernatant, and centrifuge at 100,000 × *g* for 1 h at 4 °C. Dialyze the final supernatant at 4 °C in transport buffer with three changes of the buffer. After dialysis, determine the concentration of protein and adjust it to 10 mg/ml by dilution with transport buffer or by concentration with a Centricon concentrator (Amersham). Aliquot, freeze in liquid nitrogen, and store at −80 °C.
2. Second, prepare PICs for nuclear import analysis. For preparation of cytosol of HIV-infected cells, we use protocol described by Fassati and Goff [35] with several modifications. Approximately $10^7$ HeLa-CD4 cells infected with HIV-1 NL4-3 in the presence of Polybrene (8 μg/ml) for 16 h are washed with PBS–0.5 mM EDTA, trypsinized, and washed again with PBS. All subsequent manipulations are carried out at 4 °C. Cells are resuspended in 5 volumes of hypotonic buffer, centrifuged, and resuspended in 3 volumes of hypotonic buffer supplemented with 0.025 % Brij 96 to disrupt PIC association with the

[1]Digitonin concentration of 40 μg/ml is usually sufficient for this purpose.

cytoskeleton. Following incubation for 10 min at 4 °C, cells are homogenized with 10–15 strokes in a Dounce homogenizer, and nuclei and unbroken cells are pelleted by centrifugation at 3,300 × *g* for 15 min. The supernatant (cytoplasmic extract) is clarified by centrifugation at 7,500 × *g* for 20 min, and the pellet is discarded. Viral PICs are purified from cytoplasmic extract by centrifugation through a 45 % sucrose cushion (in hypotonic buffer) at 34,000 rpm (100,000 × g) in a Beckman SW-60 rotor for 3 h at 4 °C. Pellets of HIV-1 PICs are resuspended in 200 μL of HeLa cytosol, snap-frozen in liquid $N_2$, and stored at −80 °C.

3. Grow HeLa cells to 70 % confluency and wash with serum-free medium. Other cell types can also be used.

4. Permeabilize cells by incubating them for 5 min at 4 °C in serum-free mixed medium supplemented with 40 μg/ml of digitonin (Fluka) added immediately before use.

5. An aliquot of cells is used to test for the intactness of nuclei by an assay based on differential staining of native and compromised nuclei with FITC-WGA or FITC-ConA (100 μg/ml). Staining is observed on a fluorescent microscope (*see* Note 2).

6. After permeabilization, wash cells twice with ice-cold transport buffer.

7. Harvest cells using a plastic scraper, and resuspend in transport buffer at $5 \times 10^6$ cells/ml.

8. For each assay, 34 μl of cells are incubated for 1 h at 30 °C in a 100 μl reaction containing cytoplasmic lysate from HIV-infected cells (any HIV-infected cell cytosol can be used) supplemented with energy regenerating system and dNTPs (0.1 mM). Individual nuclear import factors can be eliminated from the lysate by incubation with specific antibodies attached to Sepharose beads. It is also possible to recreate nuclear import process by mixing purified PICs, recombinant nuclear import factors (importins, Ran, NTF2), and energy [34].

9. Negative controls should be included to demonstrate that observed nuclear import is physiologically relevant. These negative controls are cytosol without an ATP-regenerating system and cytosol with ATP-regenerating system and 0.8 mg/ml wheat germ agglutinin (WGA) (*see* Note 3).

10. At the end of incubation, wash the cells twice with ice-cold transport buffer.

11. Cells can be fixed with 1 % formaldehyde in transport buffer for 30 min on ice and analyzed by fluorescent microscopy (if fluorescently tagged PIC was used) or DNA can be extracted and subjected to PCR analysis using primers specific for 2-LTR circle forms of the HIV-1 DNA or integrated HIV-1 provirus [36].

[2]FITC-WGA binds to uncompromised nuclei, whereas FITC-ConA binds to damaged nuclei [44].
[3]WGA binds to nucleoporins and inhibits nuclear import.

### 3.2 Analysis of VpR-Induced G2 Arrest in Synchronized Mammalian Cells (See Note 4)

1. HeLa cells are grown in Dulbecco's modified Eagle's medium (DMEM, Cellgro) supplemented with 10 % fetal bovine serum (FBS, Invitrogen). HeLa cells are first synchronized to the G1/S boundary of the cell cycle using a previously described double thymidine (DT) block method [37]. Specifically, HeLa cells are treated with 2 mM thymidine for 19 h, washed three times with PBS, and then placed in DMEM for 8 h. 2 mM thymidine is added again for 16 h, blocking all cells in the G1/S boundary.

2. Prior to adenoviral infection, synchronized cells are washed three times with PBS and placed in DMEM. Cells are then infected with VpR-expressing (Adv-VpR) or control (Adv) adenoviral vector with 1.0 MOI (multiplicity of infection).

3. Adv- or Adv-VpR transduced cells are collected at 0, 5, 8, and 11 h after viral transduction by trypsinization, the time points corresponding to cell progression through G1/S, S, G2, and G1, respectively. Cells are then washed twice with 2 ml of 5 mM EDTA/PBS and centrifuged at 500 × *g*. After resuspension in 1 ml of 5 mM EDTA/PBS, cells are fixed with 2.5 ml of 95–100 % cold ethanol and kept at 4 °C overnight. After centrifugation, fixed cells are washed twice with 2 ml of 5 mM EDTA/PBS and centrifuged at 500 × *g*. After resuspending in 0.5 ml PBS, cells are incubated with RNase A (50 μg/ml) at 37 °C for 30 min and then at 0 °C with addition of propidium iodine (PI, 10 μg/ml) for 1 h.

4. Cells are finally filtered prior to analysis of DNA content by 'flow cytometry on a FACScan cytometer (Becton Dickinson). The cell cycle profiles are then modeled by using the ModFit software (Verity Software House, Inc.).

### 3.3 Analysis of VpR-Induced G2 Arrest in Fission Yeast

The use of fission yeast (*S. pombe*) as a model system to study Vpr-induced G2 arrest has revealed many features of VpR-induced G2 arrest. For example, it was shown that VpR induces G2 arrest specifically through Tyr15 hyperphosphorylation of Cdc2/CDK1, which is the cyclin-dependent kinase that determines onset of mitosis in all eukaryotic cells [12, 14, 32]. The advantage of studying the effect of VpR on cell cycle G2 arrest in fission yeast is the possibility to express HIV-1 *vpr* gene under control of an inducible *nmt1* (no message in thiamine) promoter [38, 39]. This strongly regulated promoter allows to turn *vpr* gene expression OFF or ON simply by adding or removing thiamine from the growth media. This feature is quite useful because VpR is very toxic to many cells. VpR-induced G2 arrest can be specifically measured in fission yeast based on a number of cellular endpoints including cell elongation, septation index, and flow cytometric analysis [31, 32]. For initial and quick observation, VpR-induced G2 arrest is indicated by cell elongation, which is normally the result of a cell cycle G2/M delay or arrest and is commonly known as the "cdc phenotype"

[4]By using this experimental procedure, we have demonstrated for the first time that VpR induces cell cycle G2 arrest through an S phase-dependent mechanism [26], i.e., even though VpR stops the cell cycle at the G2/M phase, the initiation events such as Chk1-$Ser^{345}$ phosphorylation actually occur in the S phase of the cell cycle. Future mechanistic studies should include analysis of cell cycle S phase-specific cellular proteins that interact with VpR and might be responsible for responses to VpR and induction of cell cycle G2 arrest.

[31, 40, 41]. In thiamine-containing growth medium (*vpr*-OFF), fission yeast cells with *vpr* plasmid are of normal length, which ranges from 7 to 12 μm [42]. In contrast, in the thiamine-free medium (*vpr*-ON), cell length becomes significantly longer than of normal cells, indicating growth delay at G2/M boundary of the cell cycle induced by VpR [32]. As an alternative, forward scatter analysis can be used to detect both cell elongation and gross enlargement of the *vpr*-expressing cells in a cell population of $10^4$ [32, 39]. To quantify the degree of VpR-induced G2 arrest, flow cytometric analyses are normally used to measure the cell cycle profile by DNA content [32]. Typically, 70–80 % of the synchronized G1 cell population in *vpr*-repressing cells will shift to the G2 phase as soon as *vpr* is expressed. As an alternative measurement, VpR-induced G2 arrest in the yeast system can also be confirmed by septation index analysis, which measures the percent of cells passing mitosis as shown by formation of a septum between the dividing daughter cells, an indication of cell cycling [43]. Normally 10–15 % of cells contain septa in an actively growing *S. pombe* population that decreases to zero when *vpr* is expressed [32].

## Acknowledgments

This work was supported in part by the District of Columbia Developmental Center for AIDS Research (DC D-CFAR), NIH-funded program (1P30AI087714 – 01, MB), and NIH-NINDS-R21-NS063880 (RZ). The authors would like to thank Ge Li for technical assistance of this manuscript.

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