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Vpr-Host Interactions during HIV-1 Viral Life Cycle

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Abstract

HIV-1 viral protein R (Vpr) is a multifunctional viral protein that plays important role at multiple stages of the HIV-1 viral life cycle. Although the molecular mechanisms underlying these activities are subject of ongoing investigations, overall, these activities have been linked to promotion of viral replication and impairment of anti-HIV immunity. Importantly, functional defects of Vpr have been correlated with slow disease progression of HIV-infected patients. Vpr is required for efficient viral replication in non-dividing cells such as macrophages, and it promotes, to some extent, viral replication in proliferating CD4+ T cells. The specific activities of Vpr include modulation of fidelity of viral reverse transcription, nuclear import of the HIV-1 pre-integration complex (PIC), transactivation of the HIV-1 LTR promoter, induction of cell cycle G2 arrest and cell death *via* apoptosis. In this review, we focus on description of the cellular proteins that specifically interact with Vpr and discuss their significance with regard to the known Vpr activities at each step of the viral life cycle in proliferating and non-proliferating cells.

Keywords

HIV-1; Viral protein R (Vpr); Macrophages; Reverse Transcription; Nuclear transport; Transcriptional Activation of LTR promoter; G2 Arrest; Apoptosis

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 highly conserved among HIV, simian immunodeficiency viruses (SIV) and other lentiviruses (Tristem et al., 1992; Tristem et al., 1998). Besides lentiviruses, the Vpr protein sequence shares no strong homology with any other known protein. A tertiary structure of Vpr proposed based on the NMR analysis (Fig. 1) consists of an α-helix-turn-α-helix domain in the amino-terminal half from amino acids 17 to 46, and a long α-helix from aa 53

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to 78 in the carboxy-terminal half (Schuler et al., 1999; Wecker and Roques, 1999). These three α-helices are folded around a hydrophobic core in a structure which allows interaction of Vpr with different cellular proteins (Morellet et al., 2003). These interactions underlie the specific roles of Vpr during the HIV-1 viral life cycle.

Increasing evidence suggest that HIV-1 Vpr plays a significant role in viral pathogenesis. For example, some of the earlier studies in SIV-infected Rhesus monkeys suggested that depletion of the SIV *vpr* and *vpx* genes, two homologous counterparts of the HIV-1 Vpr, severely sabotaged the ability of SIV to cause AIDS (Gibbs et al., 1995; Lang et al., 1993). The requirement of Vpr in viral survival and pathogenesis was further supported by the finding that a mutated *vpr* gene reverted to the wild type in both infected chimpanzees and a human subject (Goh et al., 1998). Conversely, viruses in some patients with slow disease progression were shown to carry functionally defective Vpr (Caly et al., 2008; Goh et al., 1998; Somasundaran et al., 2002; Zhao et al., 2002).

Vpr displays a number of unique activities in host cells during the HIV-1 viral life cycle (Fig. 2). These include nuclear transport of PIC (Heinzinger et al., 1994b), activation of the HIV-1 LTR promoter (Felzien et al., 1998; Kino et al., 2002), induction of cell cycle G2 arrest (He et al., 1995; Li et al., 2007; Re et al., 1995), and induction of apoptosis (Stewart et al., 1997).

In this review, we summarize our current understanding of each of these Vpr activities by focusing specifically on the cellular proteins that interact with Vpr. The functional relevance of these Vpr-protein interactions and their impact on viral replication in proliferating and non-proliferating cells is discussed.

Role of Vpr in HIV-1 reverse transcription

Upon penetration of HIV into the host cell, the nucleocapsid of the virus is released into the cytoplasm of the host cell, where reverse transcription takes place to convert viral RNA to proviral DNA. In HIV-1, initiation of reverse transcription requires the $tRNA^{Lys3}$-mediated priming (Huang et al., 1994). Importantly, to function as a primer for reverse transcription, $tRNA^{Lys3}$ must have a free 3′ end, i.e. be deacetylated. Vpr appears to promote incorporation of deacetylated $tRNA^{Lys3}$ into assembling viral particles because it specifically binds to the Lys-tRNA synthetase, which acetylates $tRNA^{Lys3}$, and inhibits its activity (Stark and Hay, 1998).

HIV-mediated reverse transcription is an error-prone process, which generates on average one mutation out of 2,000 to 5,000 polymerized nucleotides it transcribes (Li et al., 1997; Romani and Engelbrecht, 2009). These mutations are generated during reverse transcription to a large extent by accidental incorporation of dUTP or deamination of cytosine that yields uracil (Chen et al., 2004; Mansky et al., 2000). If the uracil is not properly removed from the viral DNA by uracil-N-glycosylase (UNG; also known as UDG) or dUTPase, it will lead to transitional mutations that convert Cytosine (C) to Thymine (T) on one strand, and Guanine (G) to Adenine (A) on the other strand.

One of the advantages for the virus to have such an error-prone reverse transcriptional process is to generate a pool of highly diversified viral genomes that can quickly adapt to an adverse host environment such as that in patients who are receiving highly active antiretroviral therapies (HAART). This is one of the reasons why drug resistant HIV can emerge rapidly in HAART-treated patients. On the other hand, too many viral mutations can also be detrimental to the virus. For instance, mis-incorporation of uracil into viral DNA could result in viral DNA degradation or inhibition of initiation of viral DNA synthesis (Klarmann et al., 2003). Therefore, a fine balance between generation of viral genomic diversity and production of functionally defective viral DNA could potentially be a critical factor for viral survival.

Vpr appears to play a specific role in viral reverse transcription via a strong interaction with the nuclear form of uracil-N-glycosylase (UNG2) (Bouhamdan et al., 1996). UNG2 is a DNA excision DNA repair enzyme that specifically removes uracil from nuclear DNA (Parikh et al., 2000). However, it is not totally clear at the moment what the role of Vpr is in this process. One school of thought is that Vpr augments the UNG2 activity, i.e., it promotes increased fidelity of the viral reverse transcription by recruiting UNG2 to the viral particles in order to proof-read or remove uracil from viral DNA. Evidence to support this notion includes Vpr-mediated incorporation of UNG2 into the HIV-1 viral particles (Chen et al., 2004). Consistent with this notion, in the absence of Vpr, viral mutational rates increase at least 4- or 18-fold in proliferating T-cells and non-dividing macrophages, respectively (Chen et al., 2004; Chen et al., 2002; Mansky et al., 2000). In contrast to a positive role of Vpr in supporting the UNG2 activity, others suggest that Vpr may antagonize the UNG2 activity, i.e., UNG2 is a host antiviral factor and the Vpr-UNG2 interaction is intended to reduce the number of abasic sites in the viral DNA genome that are generated after the removal of uracil by UNG2 (Fenard et al., 2009; Schrofelbauer et al., 2005). Thus, Vpr may actually counteract the UNG2 effect by promoting viral survival or genomic diversity by preventing virion incorporation of UNG2 through proteasome-mediated degradation (Ahn et al., 2010; Schrofelbauer et al., 2005). This notion is also consistentwith a recent report showing that overexpression of UNG2 inhibits HIV-1 replication through reduced viral transcription (Fenard et al., 2009). Interestingly, the function of Vpr-UNG2 interaction seems to duplicate the function of the Vif-APOBEC3G interaction. The cellular antiviral factor APOBEC3G, a cellular deaminase, destroys viral transcripts by creating uracil in viral DNA genome through deamination of cytosine residues during reverse transcription; Vif counteracts this antiviral effect by preventing virion incorporation of APOBEC3G through proteasome-mediated proteolysis (Mariani et al., 2003; Sheehy et al., 2002). Similarly, assuming that UNG2 is indeed an antiviral factor, by removing uracil UNG2 could generate sufficient numbers of abasic sites in the viral transcripts to cause destruction of the viral genome; Vpr counteracts the effect of UNG2 by preventing virion incorporation of UNG2 *via* proteasome-mediated protein destruction. Moreover, uracil is the common theme and is manipulated in the viral particles by both APOBEC3G and UNG2. There might be even an interrelationship between the Vpr-UNG2 and the Vif-APOBEC3G effects, as suggested by a report showing that Vpr enhances protein production of Vif (Li et al., 2008).

Although it is clear that Vpr plays an important role in viral reverse transcription, the specific function of Vpr in the Vpr-UNG interaction during this viral infection step awaits resolution.

Nuclear transport of HIV-1 preintegration complex (PIC)

One of the unique Vpr activities is its ability to shuttle between the cytoplasm and the nucleus (Heinzinger et al., 1994a), which is believed to contribute to nuclear import of the viral preintegration complex (PIC) (de Noronha et al., 2001; Heinzinger et al., 1994a; Popov et al., 1998b). 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 (Connor et al., 1995; Heinzinger et al., 1994a). Other viral proteins involved in the nuclear transport process of PIC include Matrix Antigen (MA) (Bukrinsky et al., 1993) and integrase (IN) (Gallay et al., 1997). The involvement of Vpr in nuclear transport of PIC was further supported by co-localization of Vpr and PIC within 4–16 hrs after onset of viral infection (Fassati and Goff, 2001).

In normal cells, nuclear transport of a particular protein 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 from the nuclear envelope. The imported protein usually carries a nuclear localization sequence (NLS) domain that consists of a short region of basic amino acids (lysines and arginines) or 2 such regions spaced about 10 amino acids apart (Nakielny and Dreyfuss, 1999; Wente, 2000). Typically, the importin-α binds the NLS-containing cargo and serves as a bridge between the cargo and the receptor importin β through the importin β-binding domain (IBB) on importin α. The transporting process involves docking of the ternary protein complex to the nuclear envelope, translocation through the nuclear pore, and release of the cargo protein into the nucleoplasm (Herold et al., 1998; Kobe, 1999).

Currently, there are three hypotheses, which are not necessarily mutually exclusive, that could potentially explain the mode of action of Vpr in nuclear transport of the HIV-1 PIC. The first model hypothesizes that Vpr targets the HIV-1 PIC to the nucleus via a distinct, importin-independent pathway (Gallay et al., 1996; Jenkins et al., 1998); the second suggests that Vpr modifies cellular importin-dependent import machinery (Popov et al., 1998a; Popov et al., 1998b); and the third implies that importin α alone is sufficient for Vpr-mediated nuclear import activity.

The first model was based on the observation that in the *in vitro* nuclear import assay Vpr can enter nuclei in the absence of soluble import factors (Jenkins et al., 1998). Consistent with this concept, Vpr was shown to induce dynamic disruptions in the nuclear envelope (de Noronha et al., 2001) which may serve as entry points for isolated Vpr and for the PICs. HIV-1 Vpr has been shown to co-precipitate with fission yeast nucleoporin Nup124p and its human homolog, NUP153, and nuclear import of Vpr was impaired in nup124 null mutant strain (Varadarajan et al., 2005). Vpr also interacts with human nucleoporin CG1, which contributes to Vpr docking to the nuclear envelope (Le Rouzic et al., 2002). Therefore, Vpr may function as a substitute for importin β, which also interacts with nucleoporins to

mediate nuclear translocation of its cargo (Pemberton and Paschal, 2005). Consistent with this notion, Vpr was shown to interact specifically with nucleoporin phenylalanine-glycine (FG)-repeat regions, critical for importin-mediated nuclear import (Fouchier et al., 1998).

The second model postulates that Vpr uses a modification of the importin α,β-dependent pathway to enter the nucleus (Bukrinsky, 2004). Vpr was shown to bind to importin α both from human and budding yeast cells, but the binding site is different from the binding site for NLS (Agostini et al., 2000; Popov et al., 1998a; Popov et al., 1998b; Vodicka et al., 1998). This binding of Vpr to importin α appears to stimulate subsequent nuclear import of the cargo (Popov et al., 1998b), likely by increasing the affinity of NLS-importin α interaction (Agostini et al., 2000). The effect of Ran-GTP binding to importin β on the ternary complex has not been reported and it is not understood why Vpr is frequently observed to localize at the nuclear envelope, although this may be related to the binding of Vpr to nucleoporins described above. Another possibility is that Vpr may localize on the nuclear envelope via interaction with the 26S proteasome (Li et al., 2010a). In fission yeast, the 26S proteasome complex attaches to the inner side of the nuclear membrane (Wilkinson et al., 1998). Consistent with this idea, Vpr fell off the nuclear membrane when the 26S proteasome was released from the nuclear membrane in a temperature-sensitive *cut8* mutant (Li et al., 2010a). Even though it is unclear where the 26S proteasome localizes in mammalian cells, one study found Vpr to be at the inside of the nuclear envelope (Vodicka et al., 1998). This suggests that Vpr may be transported through the pore but may not be released into the nucleoplasm.

The third model suggests that importin α alone may be sufficient for the nuclear transport activity of Vpr, without utilizing the classical importin β-dependent transport pathway (Nitahara-Kasahara et al., 2007). In support of this hypothesis, depletion of importin α from HeLa cells by using siRNAs markedly decreased the nuclear import of Vpr in an *in vitro* nuclear transport assay, whereas no effect was seen when importin β was depleted from the cell extracts (Nitahara-Kasahara et al., 2007). A similar importin α-driven mechanism was also observed in macrophages. The requirement of importin α in Vpr-mediated nuclear transport of PIC and viral replication in macrophages was further demonstrated by a significant defect in replication of the virus carrying mutations in the first α-helix region of Vpr critical for interaction with importin α (Nitahara-Kasahara et al., 2007). Thus, expression of importin α and importin α-driven nuclear import of Vpr are essential for efficient viral replication in macrophages.

The ability of HIV-1 to infect and replicate in non-dividing cells (terminally differentiated macrophages and incompletely activated CD4+ T lymphocytes) is the characteristic feature of lentiviruses that determines to a large extent their high replicative capacity and pathogenesis. Moreover, the ability of Vpr to promote nuclear transport of PIC is generally accepted as the reason why Vpr is required for efficient viral replication in non-dividing cells such as macrophages (Connor et al., 1995; Di Marzio et al., 1995; Heinzinger et al., 1994b; Subbramanian et al., 1998). However, a recent paper argued against this explanation by showing that infection of growth-arrested T-cells by Vpr(−) HIV-1 was reduced by 2-fold compared to the wild-type virus (Yamashita et al., 2007), which was essentially the same level of reduction observed in proliferating cells. In addition, Vpr likely participates in

nuclear import of PIC in T cells in a similar manner as it does in macrophages, and nuclear import through the nuclear pore is essential for HIV replication in both cell types (BouHamdan et al., 1998; Bukrinsky and Haffar, 1997; Riviere et al., 2010). Therefore, the differential contribution of Vpr to viral replication in macrophages and T-cells can be attributed neither to the proliferation status of the target cell nor to the ability of Vpr to promote nuclear import in non-dividing cells.

Other evidence suggests that the differences in Vpr's contribution to viral replication in different cell types could be due to the presence of different cellular factors that influence Vpr's activity. For example, HSP70 present in T-cells can substitute for the activity of Vpr thus diminishing dependence of viral replication on Vpr in T-cells. This is in sharp contrast to macrophages where HSP70 is less abundant (Agostini et al., 2000) and Vpr is required for efficient viral replication (de Noronha et al., 2001; Heinzinger et al., 1994a; Popov et al., 1998b). Vpr co-precipitates with HSP70 in both proliferating T-cells and macrophages (Iordanskiy et al., 2004a; Iordanskiy et al., 2004b). While HSP70 minimizes the Vpr dependence in proliferating T-cells, the direct Vpr-HSP70 interaction creates functional competition between these two proteins. For example, HSP70 inhibits viral replication in macrophages in the presence of Vpr but stimulates nuclear transport and viral replication in the absence of Vpr (Iordanskiy et al., 2004a).

In summary, at least three different models can potentially explain the possible role of Vpr in nuclear transport of PIC. Interactions of Vpr with different cellular proteins such as importin α, NUP153, CG1 and HSP70 may represent different underlying molecular mechanisms occurring in different cell types under different circumstances.

Activation of HIV-1 LTR-mediated transcription

Once the HIV-1 proviral DNA is integrated into the human chromosomes, Vpr promotes HIV-1 viral gene transcription by direct interaction with the long terminal repeat (LTR) promoter. The Vpr-mediated LTR gene transcription is achieved by association of Vpr with various transcriptional factors or co-factors on the LTR promoter (Table 1). The electrophoretic mobility shift analysis (EMSA) and other DNA-protein binding assays suggested direct binding of Vpr to the LTR DNA sequences that span a number of transcriptional binding sites on LTR including the NF-kB, SP1, p300/CBP binding sites and the adjacent C/EBP sites (Hogan et al., 2003). Because Vpr interacts with these highly conserved transcription factor binding sites, besides LTR it also enhances gene transcription of other related promoters, such as mouse mammary tumor virus (MMTV) promoter (Kino et al., 2002) and CMV or SV40 promoters (Roux et al., 2000).

One of the Vpr trans-activating activities on LTR is through promoting phosphorylation of IkB, which leads to binding of NF-kB to Vpr *via* its LxxLL motif (Northrop et al., 1992), nuclear translocation of NF-kB, and subsequent binding of NF-kB to the LTR response element resulting in NF-kB and SP1-mediated increase of gene transcription (Varin et al., 2005). Another Vpr trans-activating activity on LTR includes the interaction of Vpr with the transcriptional factor IIB (TFIIB) (Agostini et al., 1996). Follow-up studies demonstrated that Vpr may activate transcription by promoting conformational changes of TFIIB

(Agostini et al., 1999). Vpr also promotes LTR gene transcription by forming a stable complex with p300/CBP (a CREB-binding protein) and the ligand-bound GR through the glucocorticoid response element (GRE) (Felzien et al., 1998; Kino et al., 2002). Here Vpr acts as an adapter that links p300/CBP and GR coactivator for LTR gene transcription (Kino, 2002). Interestingly, mutation analysis showed that p300/CBP binds to the C-terminal end of Vpr, which is a critical region for induction of cell cycle G2 arrest. This finding suggests a possible association of Vpr-mediated cell cycle G2 arrest and viral gene transcription. Consistently, Vpr is in the same protein complex with p300 and cellular protein p21 (WAF1), which alleviates p21-mediated inhibition of cell departure from G1 phase (Cui et al., 2006). A recent study showed that the entry of Vpr-producing cells from G1 to the S phase triggers an S-phase dependent induction of cell cycle G2 arrest by Vpr (Li et al., 2010b). It is conceivable, therefore, that the regulation of p300 by Vpr ensures transition of HIV-1 infected cells from G1 to S and ultimately results in growth arrest in the G2 phase of the cell cycle. This scenario may also provide a potential mechanism to explain enhanced viral replication in proliferating cells after growth arrest in the G2 phase (Felzien et al., 1998; Goh et al., 1998).

In monocytes and macrophages, binding of Vpr to the LTR C/EBP binding region and its adjacent regions including the NF-kB site is required for subtype B HIV-1 gene expression (Liu et al., 1999). Vpr promotes C/EBP binding to LTR either indirectly or jointly to form a complex (Hogan et al., 2003; Kilareski et al., 2009). Interestingly, there is a sequence variation in the HIV-1 C/EBP binding site. It appears that Vpr has stronger binding to the C/EBP binding site I variants, which correlates with the late stage of HIV-1 associated disease. Consistently, increased binding of Vpr to the C/EBP binding site I variants within LTR was found to correlate with HIV-1 associated dementia (Burdo et al., 2004).

Altogether, Vpr promotes HIV-1 LTR gene transcription through interactions with various transcriptional factors and co-factors in both proliferating T-cells and non-dividing macrophages. In addition, this transacting effect on LTR is synergistic with the effect of HIV-1 Tat that also promotes LTR gene transcription (Sawaya et al., 2000).

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 (He et al., 1995; Jowett et al., 1995; Re et al., 1995). The cell cycle G2 arrest induced by Vpr is thought to suppress human immune function by preventing T-cell clonal expansion (Poon et al., 1998) and to provide an optimized cellular environment for maximal levels of viral replication (Goh et al., 1998). However, contribution of Vpr to viral replication in proliferating T-cells is relatively small (Goh et al., 1998; Iordanskiy et al., 2004a); 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 unclear.

Induction of cell cycle G2 arrest by Vpr is a highly conserved activity as the same effect has been observed also in other eukaryotes such as fission yeast (*Schizosaccharomyces pombe*) (Masuda et al., 2000; Zhao and Elder, 2005; Zhao et al., 1996; Zhao and Elder, 2000). For

example, Vpr induces cell cycle G2 arrest specifically through Tyr15 hyperphosphorylation of Cdc2/CDK1, which is the cyclin-dependent kinase that determines onset of mitosis in all eukaryotic cells (He et al., 1995; Re et al., 1995; Zhao et al., 1996). Consistently, Vpr induces G2 arrest by inhibiting the Cdc25 phosphatase through direct interaction and activation of the Wee1 kinase; both Cdc25 and Wee1 enzymes directly regulate Cdc2/CDK1 (de Noronha et al., 2001; Elder et al., 2001; Goh et al., 2004; Kino et al., 2005; Masuda et al., 2000).

One of the recent developments in understanding Vpr-induced G2 arrest is the involvement of ubiquitin-proteasome system (UPS). In particular, a specific Cullin ubiquitin E3 ligase known as Cul4A-DDB1-DCAF1/VprBP was found to be associated specifically with this G2 induction process (Belzile et al., 2007; DeHart et al., 2007; Hrecka et al., 2007; Le Rouzic et al., 2007; Schrofelbauer et al., 2007; Tan et al., 2007; Wen et al., 2007). This E3 ligase association is mediated through direct interaction of VprBP with Vpr on chromatin (Belzile et al., 2010a; Zhang et al., 2001). The identification of VprBP-associated E3 as a contributor to Vpr-induced G2 arrest implicates a role of Vpr in protein polyubiquitination (poly-Ub) and proteasome-mediated proteolysis. Indeed, Vpr promotes the E3 ligase activity (Hrecka et al., 2007) and promotes protein polyubiquitination through the K48 linkages (Belzile et al., 2010b). Inhibition of polyubiquitination by Ub(K48R) or suppression of proteasome activity by epoxomicin or MG132 reversed Vpr-induced G2 arrest (DeHart et al., 2007; Tan et al., 2007). Moreover, Vpr is now known to associate directly with the 26S proteasome through an hHR23A-mediated mechanism (Li et al., 2010a). However, the downstream cellular protein(s) that are targeted by Vpr-mediated UPS for the G2 induction are still unknown Recently, CDC25C was shown to be one of this downstream effectors during Vpr-induced cell cycle G2 arrest (Li et al., 2010b).

It is worthwhile to note that the recent finding on hHR23A-mediated interaction of Vpr with 26S proteasome may shed new light on an old unresolved story of the specific role that HHR23A plays in the Vpr activities. hHR23A is a member of a highly conserved excision DNA repair Rad23 protein family that contains an N-terminal ubiquitin-like (UbL) and two C-terminal ubiquitin-associated (UbA) domains (Elder et al., 2002b; Gragerov et al., 1998; Withers-Ward et al., 1997). Earlier studies showed that Vpr binds to hHR23A through its C-terminal UbA domain (Dieckmann et al., 1998). Interaction of Vpr with hHR23A was thought to play a role in the induction of G2 arrest (Gragerov et al., 1998; Withers-Ward et al., 1997). However, subsequent mutational analysis could not relate this interaction to any known biological function of Vpr (Mansky et al., 2001). A recent report showed that hHR23A serves as a bridging protein to associate Vpr with proteasome and this function is essential for the stimulatory effect of Vpr on HIV-1 replication in growth-arrested HeLa cells and macrophages (Li et al., 2010a). Since new evidence suggests that Vpr induces G2 arrest through UPS, it is possible that hHR23A may also be involved in induction of cell cycle G2 arrest through Vpr-mediated proteolysis of downstream cellular proteins *via* UPS. This possibility remains to be tested.

A major question in studying Vpr-induced G2 arrest is the actual cause of this arrest. Is it a consequence of host cell cycle checkpoints modified by Vpr? Or is it the result of Vpr's active actions in modulating host cell cycle regulation? The main confusion came from the

fact that some of the host cell cycle checkpoint proteins are involved in this process. 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 regulated by CDC25 or WEE1. Thus, it is logic to think that Vpr might induce G2 arrest through one of these two checkpoint pathways (for detailed reviews, see (Amini et al., 2004; Andersen et al., 2008; Elder et al., 2002a; Zhao and Elder, 2005; Zhao and Elder, 2000)). Consistent with this notion, Vpr induces DNA double-strand breaks (DSBs), which supports the idea that Vpr induces G2 arrest through DNA damage checkpoint (Tachiwana et al., 2006). However, expression of *vpr* does not change the radiosensitivity of the checkpoint defective mutants (Elder et al., 2000) or increase gene mutation frequency (Mansky, 1996), which argues against the possibility that Vpr actually causes DNA damage. Similarly, another report showed that Vpr does not induce DNA DSBs (Lai et al., 2005). Moreover, down-regulation of H2AX, the 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 (Li et al., 2007). In addition, the ATR kinase, rather than the ATM kinase involved in cell cycle arrest in response to double-strand DNA breaks, was found to play a major role in Vpr-induced G2 arrest through activation of Chk1 *via* S345 phosphorylation (Li et al., 2007; Li et al., 2010b; Roshal et al., 2003; Zimmerman et al., 2004). 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 pathway activated by Vpr and by hidroxyurea/UV light. 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 (Lai et al., 2005; Roshal et al., 2003; Zimmerman et al., 2004), all of which indicate activation of DNA replication checkpoint control. However, these conclusions remain unsatisfactory because activation of DNA replication checkpoint generally leads to S phase arrest but not G2 arrest.

One of the possible attributing 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 hrs after introduction of Vpr in an asynchronized cell population. With this single late time point, it is not possible to establish the sequence of events to determine which events cause the G2 arrest, and which events occur after the initiation of G2 arrest, such as DSBs, and therefore are not responsible for the G2 arrest. Therefore, defining the temporal order of events is critical for identifying the cause of the G2 arrest. Characterization 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 purpose, we have adapted an approach that allows us to monitor the cellular signaling for Vpr-induced G2 arrest within eleven hours of a single cell cycle (Li et al., 2010b). Surprisingly, results of this study suggested that Vpr induces cell cycle G2 arrest through an S phase-dependent mechanism (Li et al., 2010b), i.e., even though Vpr stops the cell cycle at the G2/M phase, the initiation event, such as Chk1-Ser^{345} phosphorylation, actually occurs in the S phase of the cell cycle. Subsequent mechanistic characterization suggested that the triggering cellular signal(s) in

the S phase of the cell cycle include, at least in part, a DNA licensing factor Cdt1 (Li et al., 2010b).

Overall, accumulating evidence suggest that Vpr-induced G2 arrest is unique in many aspects and different from activation of the DNA damage or DNA replication checkpoint. This notion is supported by several findings. First, DNA damage and replication checkpoint control mechanisms are highly conserved among eukaryotes. However, Vpr is still able to induce G2 arrest in mutant fission yeast strains that are defective in the DNA damage and/or replication checkpoints, arguing that these cell cycle checkpoint machineries were not activated in Vpr-induced cell cycle arrest in fission yeast (Elder et al., 2000; Matsuda et al., 2006). Second, both fission yeast and mammalian protein phosphatase 2A (PP2A) are uniquely required for induction of Chk1-Ser^{345} phosphorylation and Vpr-induced G2 arrest (Elder et al., 2001; Li et al., 2007; Masuda et al., 2000). Significantly, this PP2A-dependent Chk1-Ser^{345} phosphorylation is not required for hydroxyurea (HU) or ultraviolet light (UV) induced cell cycle arrest, even though both of them cause the same ATR-dependent Chk1-Ser^{345} phosphorylation through activation of DNA replication checkpoint control. Third, the cellular signal(s) that lead to G2 arrest is initiated through Chk1-Ser^{345} phosphorylation at the S-phase of the cell cycle. Even though HU and UV also induce Chk1-Ser^{345} phosphorylation in the S phase under the same conditions, neither HU nor UV-treated cells were able to pass through the S phase, whereas *vpr*-expressing cells completed the S phase and stopped at the G2/M boundary. Fourth, unlike HU/UV, Vpr promotes Chk1- and proteasome-mediated protein degradations of Cdc25B/C for G2 induction, and Vpr had little or no effect on Cdc25A protein degradation normally mediated by HU/UV (Li et al., 2010b).

Altogether, these data suggest that Vpr induces cell cycle G2 arrest through a unique molecular mechanism that regulates host cell cycle regulation in an S-phase dependent fashion. It is quite likely that Vpr-induced G2 arrest is an active viral action, in which Vpr utilizes the cell cycle G/M checkpoint proteins, such as ATR and Chk1, ito induce G2 arrest.

Induction of apoptosis

Vpr also causes cell death, primarily through apoptosis. It is unclear at present what the biological significance of Vpr-induced apoptosis to HIV-1 infection is. However, this cytotoxic effect may contribute to depletion of CD4+ T cells, and associates with the clinical symptoms such as dementia and the painful peripheral neuropathy of HIV-infected patients (Acharjee et al., 2010; Lum et al., 2003; Pomerantz, 2004). Molecular mechanisms underlying Vpr-induced cell death and apoptosis are complex. For example, the mitochondria-mediated apoptosis, i.e., the intrinsic apoptotic pathway, is believed to play a major role in Vpr-induced apoptosis. However, the extrinsic pathway, i.e., the receptor-mediated apoptosis, has also been reported. Thus far, there have been a number of genes and pathways proposed to have a role in Vpr-induced apoptosis, *e.g.*, direct permeabilization of the mitochondrial membranes through interaction with an adenine nucleotide translocator (ANT) or with HAX-1, the ATR-GADD45α pathway with a downstream mitochondrial role through the BAX protein, depletion of Wee1, activation of the JNK pathway or activation of the NF-κB pathway (Andersen et al., 2005; Green and Kroemer, 2004; Jacotot et al., 2000).

It is certainly possible that more than one pathway are required for full induction of apoptosis by Vpr since apoptosis pathways are often found to be redundant.

A major pathway for the induction of apoptosis by Vpr is through the mitochondria. This intrinsic pathway for apoptosis is initiated by mitochondrial membrane permeabilization (MMP) (Green and Kroemer, 2004). The release of proteins from the space between the inner and outer mitochondrial membranes ultimately leads to apoptosis. Cytochrome C is particularly important in this process since it co-operates in the cytoplasm with Apaf-1 to activate procaspase 9, the initiating caspase for the intrinsic pathway. Activated caspase 9 in turn activates the downstream caspases, such as caspase 3, which carry out many of the apoptotic events (Green and Kroemer, 2004). Vpr is thought to lead to MMP by virtue of binding to ANT protein of the inner mitochondrial membrane through its C-terminal aa71–82 domain (Brenner and Kroemer, 2003; Jacotot et al., 2001b; Jacotot et al., 2000). This binding occurs after Vpr crossing of the outer mitochondrial membrane, possibly through VDA (Voltage Dependent Anion Channel), and leads to depolarization of the inner mitochondrial membrane, swelling of the inner mitochondria and ultimately to MMP with release of the apoptosis factors. Among the considerable evidence supporting this model are depolarization of the inner mitochondrial membrane by Vpr in both fission yeast and mammalian cells (Huard et al., 2008), depolarization of isolated mitochondria by purified Vpr, strong binding between Vpr and ANT shown by several methods, reduced cell killing when ANT levels are decreased (Brenner and Kroemer, 2003; Huard et al., 2008; Jacotot et al., 2001a; Jacotot et al., 2000) and activation of caspase 9 and caspase 3 by Vpr (Muthumani et al., 2002a; Muthumani et al., 2002b; Zelivianski et al., 2006). The mitochondria-mediated apoptosis by Vpr was further supported by the anti-apoptotic activities of several cellular suppressors identified in fission yeast and mammalian cells (Huard et al., 2008; Li et al., 2009; Zelivianski et al., 2006). For example, the elongation factor 2 (EF2), a newly identified anti-apoptotic cellular factor, specifically suppresses caspase 9 and caspase 3-mediated apoptosis induced by HIV-1 Vpr (Zelivianski et al., 2006). Overexpression of fission yeast Hsp16 and Skp1 also restores mitochondria morphology in fission yeast and prevents apoptosis in mammalian cells (Benko et al., 2004; Huard et al., 2008). Interestingly, the human homologue of fission yeast Skp1, glycogen synthase kinase-3 (GSK3), appears to play both pro- and anti-apoptotic regulatory roles. GSK3 promotes mitochondria-dependent apoptosis, but it inhibits apoptosis induced by the death receptor-mediated signaling pathway (Beurel and Jope, 2006). Since GSK3 has two isoforms (α and β), it is currently unclear whether both isoforms have the same regulatory activities on apoptosis or each of the isoforms has its unique regulatory role in apoptosis. Further characterization of fission yeast Skpl, its mammalian counterparts, and their role in Vpr-induced apoptosis should provide additional insights into the important regulatory mechanisms of Skpl/GSK3 in Vpr-mediated apoptosis.

While activation of caspase-9 with no activation of caspase-8 supports the role of mitochondria-dependent induction of apoptosis by Vpr (Muthumani et al., 2002a), there have been other reports showing that Vpr activates capase-8 (Lum et al., 2003; Patel et al., 2000; Snyder et al., 2010). Caspase-8 activation is thought to be a hallmark of the extrinsic pathway for apoptosis induction by death receptors, such as FAS and TNFR1 (Barnhart and Peter, 2003). The mitochondria- and receptor-mediated apoptotic pathways were originally

considered to be separate, but later work showed that in type II cells, the apoptotic pathway initiated at death receptors by ligands such as TNFα requires amplification through the mitochondria for apoptosis to occur (Scaffidi et al., 1998; Scaffidi et al., 1999). Interestingly, a recent report showed that the C-terminal end of Vpr (aa77–92) binds to PP2A1 inducing apoptosis (Godet et al., 2010). This Vpr fragment overlaps with its mitochondrial ANT binding domain (aa71–82) that is known to induce apoptosis through mitochondria-dependent pathway.

While there is ample support for involvement of mitochondria in Vpr-induced apoptosis, there are some reports that do not readily fit into this model and which raise the possibility that Vpr may kill cells through other pathways. For instance, the localization of Vpr raises one question about the mitochondria model since Vpr has been consistently reported to be in the nucleus or at the nuclear membrane (Chen et al., 1999; Di Marzio et al., 1995; Lu et al., 1993; Mahalingam et al., 1995; Vodicka et al., 1998; Waldhuber et al., 2003) rather than in the mitochondria (Jacotot et al., 2000). It may be that only a small fraction of Vpr molecules localizes to the mitochondria, which is sufficient to induce apoptosis, and methods used to visualize Vpr may have overlooked this small amount. However, the predominant nuclear localization of Vpr and the association of nuclear localization with cell killing in Vpr mutants (Chen et al., 1999; Waldhuber et al., 2003) suggest that Vpr located in the nucleus may have some role in initiating cell killing. Consistent with this idea, Anderson et al. have presented evidence that ATR is not only responsible for the G2 arrest but has an essential role in Vpr-induced apoptosis of human cells (Andersen et al., 2005). It has also been reported that a fragment of Vpr induces cell death without caspase activation (Roumier et al., 2002), and even that Vpr induces a necrotic type of cell death in neurons (Huang et al., 2000).

Thus, the actual apoptosis inducing effect of Vpr may depend upon localization of Vpr within the cell or cellular compartments.

Summary

The activities of HIV-1 Vpr link to various steps during the HIV-1 viral life cycle, and include modulation of viral reverse transcription, nuclear transport of the PIC, transcriptional activation of the LTR promoter, induction of cell cycle G2 arrest and apoptosis. Although the molecular details of these Vpr actions are not fully understood and require further investigation, these Vpr activities are clearly executed through interactions with various cellular proteins. For example, Vpr modulates fidelity of the viral reverse transcription by direct interaction with UNG2 and SMUG. It may promote initiation of viral reverse transcription by binding to the Lys-tRNA synthetase. Even though it is debatable whether the requirement of Vpr for HIV-1 infection of non-dividing cells is due to its ability to transport the PIC into the nucleus, it is clear that the cytoplasmic-nuclear shuttling process involves associations of Vpr with numerous proteins including importin α, NUP153 and hCG1. Human heat shock proteins, such as HSP70, may in part substitute for Vpr in proliferating cells. Trans-activation of HIV-1 LTR promoter by Vpr is non-specific because Vpr promotes gene transcription of LTR through associations with a group of highly conserved transcriptional factors including SP1, p300/CBP, NF-kB, TFIIB, GR and C/EBP.

All of these transcriptional factors are also involved in activation of other cellular or viral promoters. It is evident that Vpr is a very potent cytotoxin that induces cell death and apoptosis through a rather complex mechanism but its impact depends upon where it resides in the cell and is mediated primarily through mitochondria by direct associations between Vpr and ANT, PTPC and HAX-1. Most interestingly, the interactions of Vpr with the UPS through hHR23A and the VprBP-associated E3 ligase link it both to cell cycle G2 arrest and viral replication in macrophages. However, the current challenge is to identify the cellular substrates that are specifically targeted by Vpr-induced UPS for G2 induction and viral replication. Thus, identification of these relevant substrates for Vpr mediated ubiquitination and proteolysis will help us clarify the molecular mechanisms of Vpr's activities and will provide important hints for revealing how and why Vpr is important for HIV infection in macrophages. Altogether, Vpr plays a pivotal role in viral life cycle and pathogenesis. Since Vpr activities are linked to promotion of viral infection in non-dividing macrophages and monocytes, prevention of T-cell clonal expansion, and depletion of CD4+ T-lymphocytes, future strategies to inhibit these adverse Vpr effects could potentially alleviate the impact of the viral infection and thus benefit HIV-infected patients.

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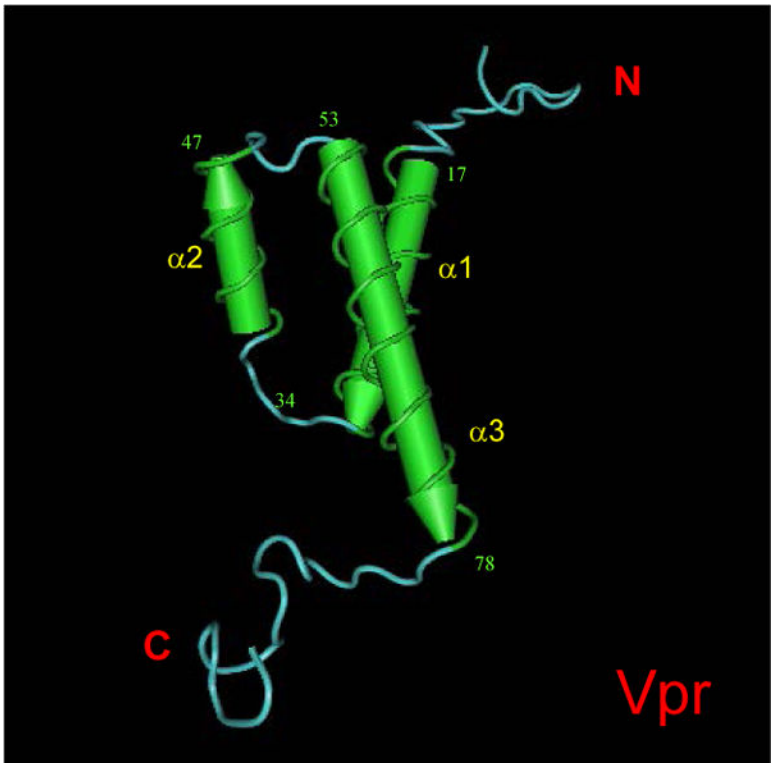

Fig. 1.
Putative tertiary structure of Vpr.

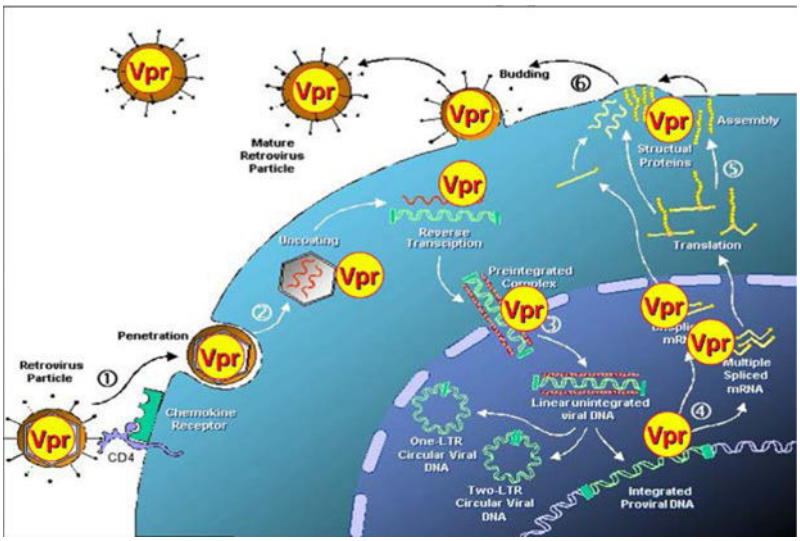

Fig. 2.

Roles of Vpr at various stages of HIV-1 viral life cycle. Vpr incorporated into the virions is released during uncoating and contributes to reverse transcription and nuclear transport of the PIC. Transactivation of HIV-1 LTR promoter, induction of cell cycle G2 arrest, and induction of apoptosis are accomplished by *de novo* produced Vpr. The specific Vpr activity at each step of the HIV-1 viral life cycle is described in the text. The figure is modified from a picture of HIV-1 life cycle provided by Bruce Patterson.

Table 1

Vpr-interacting proteins and their potential functional relevance in HIV-1 viral life cycle.

Vpr-binding protein	Protein function	Reference
Modulation of Fidelity of HIV-1 Reverse Transcription		
UNG2	Uracil DNA glycosylase	(Bouhamdan et al., 1996)
SMUG	SMUG uracil-DNA glycosylase	(Schrofelbauer et al., 2005)
Lys-tRNA synthetase	Lys tRNA synthetase	(Stark and Hay, 1998)
Nuclear Transport of Preintegration Complex		
Importin-α	Karyopherin	(Kamata et al., 2005; Popov et al., 1998b; Vodicka et al., 1998)
NUP153/Nup124	Nuclear pore proteins	(Fouchier et al., 1998; Varadarajan et al., 2005)
hCG1	Nucleoporin	(Le Rouzic et al., 2002)
HSPs (HSP27, HSP70)/Hsp16	Heat shock proteins; chaperone protein	(Benko et al., 2004; Iordanskiy et al., 2004a; Iordanskiy et al., 2004b; Liang et al., 2007)
Activation of HIV-1 LTR-mediated Transcription		
SP1	Transcription factor	(Wang et al., 1995)
p300/CBP	Nuclear receptor and transcriptional co-activator; CBP, CREB binding protein	(Felzien et al., 1998; Kino et al., 2002)
NFκB	Transcriptional factor nuclear factor kappa B	(Roux et al., 2000)
TFII B	Transcription factor	(Agostini et al., 1996)
GR	Glucocorticoid receptor	(Refaeli et al., 1995)
C/EBP	CCAAT/enhancer binding protein	(Hogan et al., 2003)
Induction of Cell Cycle G2 Arrest		
CDC25C	Protein phosphatase	(Goh et al., 2004)
WEE1	Protein kinase	(Kamata et al., 2008)
14-3-3	Adaptor protein	(Kino et al., 2005)
PP2A	Protein phosphatase 2A	(Godet et al.)
VprBP	Receptor of E3 ligase	(Zhang et al., 2001)
HHR23A/Rhp23	Excision DNA repair protein	(Gragerov et al., 1998; Li et al., 2010a; Withers-Ward et al., 1997)
Induction of Apoptosis		
ANT	Adenine nucleotide translocator	(Jacotot et al., 2001a)
PTPC	Permeability transition pore complex	(Jacotot et al., 2000)
HAX-1	Antiapoptotic mitochondrial protein	(Yedavalli et al., 2005)