The Role of Vpr in HIV-1 Pathogenesis

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Abstract: The HIV-1 *vpr* gene is conserved among the human (HIV-1, HIV-2) and simian immunodeficiency viruses (SIV). HIV-1 *vpr* encodes a 96-amino acid, 14 kDa protein (Vpr). Research from a number of laboratories in the last decade has shown that Vpr performs multiple functions, including the induction of cell cycle arrest in the G_2 phase, transactivation of the viral promoter, nuclear import of preintegration complexes, and induction of apoptosis in the infected cell. More recent studies have attempted to elucidate the cellular targets that Vpr utilizes in order to perform the above functions. This review presents the latest findings about the pathogenic events triggered by Vpr, the cellular pathways involved, and the molecular and cellular consequences of the action of Vpr in the context of HIV-1 infection.

Keywords: HIV, Vpr, G_2 arrest, apoptosis, nuclear import, transactivation.

INTRODUCTION

HIV-1 encodes four open reading frames that are dispensable for *in vitro* replication, hence their classification as “accessory genes”. One of these open reading frames was named “R” for “regulatory” (hence, viral protein R, or Vpr) after it was observed that truncation of the open reading frame would lead to a virus that replicated with slower kinetics than the wild-type counterpart [46,72,111].

The mRNA encoding HIV-1 Vpr is expressed as a late transcript during the course of infection [91]. The Vpr protein can be found in the nucleus of the infected cell, and also in association with the viral capsid *via* an interaction with Gag p6 (reviewed in [101]). *In vitro*, Vpr exists as an oligomer and its N terminus is necessary for oligomerization [115]. The precise contribution of Vpr to HIV-1 pathogenesis *in vivo* is difficult to determine, but the recent discoveries of Vpr mutations in long-term nonprogressor individuals (LTNPs) [66,96] have underscored the importance of Vpr in the viral life cycle *in vivo*.

Four functions have been ascribed to Vpr using *in vitro* systems. These are transactivation of the long terminal repeat (LTR) and certain heterologous promoters, nuclear import of preintegration complexes (PIC), induction of cell cycle arrest in G_2, and induction of apoptosis in infected cells. This review will address our current understanding of cellular pathways and mechanisms used by Vpr to accomplish these functions.

Vpr is structurally and functionally conserved in five of the primate lentiviral lineages, including HIV-1/SIVcpz, HIV-2/SIVmac/SIVsm, SIVagm, SIVsyk, and SIVmnd [100]. Vpr has also been identified in SIV isolated from other primates, such as the red-capped mangabey, and the mona and mustached monkeys [9,11,29,99], although the function of these *vpr* genes has not been determined experimentally. Interestingly, the HIV-2/SIVmac/SIVsm group encodes a gene termed *vpx*, which shares significant sequence identity with HIV-1 *vpr*. Tristem *et al*. proposed that *vpx* arose as a result of homologous recombination between SIVagm, and an ancestor of HIV-2 [100].

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EFFECTS OF VPR ON GENE EXPRESSION

The first function ascribed to Vpr was a moderate transactivation effect on the viral promoter, the LTR [23,72,89] (Fig.(**1**)). The transactivation effect exerted by Vpr is relatively modest in comparison with that of more classical viral transactivators such as HIV-1 *tat* or human T-cell leukemia virus *tax* [86]. However, two observations underscore the importance of Vpr-induced transactivation. First, the ability of Vpr to transactivate the LTR is conserved among HIV-1, HIV-2, SIVmac and SIVagm [76]. Second, experiments examining the transactivation effects of both Vpr and Tat together indicated that they are synergistic [56,89].

In addition to its role in transactivating the viral LTR, Vpr has also been shown to modulate the transcription of several cellular genes including *survivin*, and the tumor suppressor *p21^{Waf1}* [21,59,60,118]. The potential roles of *survivin* and *p21^{Waf1}* in cell survival and cell cycle arrest, respectively, will be discussed in the corresponding sections below.

Induction of G_2 arrest correlates with the ability of Vpr to upregulate transcription of the HIV-1 LTR (Fig. (**2**)). Goh *et al*. found that transcription of the HIV-1 LTR is upregulated 4-fold under conditions of G_2 arrest [42]. In addition, the loss of Vpr and the resulting decrease in transcriptional activity can be compensated for by inducing G_2 arrest by other means [42]. Furthermore, both caffeine treatment and siRNA-mediated knockdown of ATR abrogate both Vpr-induced G_2 arrest and transactivation of the LTR [87,117].

One model to explain the transcriptional effects of Vpr involves Vpr-mediated activation of the glucocorticoid receptor (GR) pathway, *via* a direct interaction between Vpr and the GR complex [83] (Fig. (**2**)). Glucocorticoids are known to play roles in controlling inflammation, and maintaining stress- and resting-related homeostasis [15,22].

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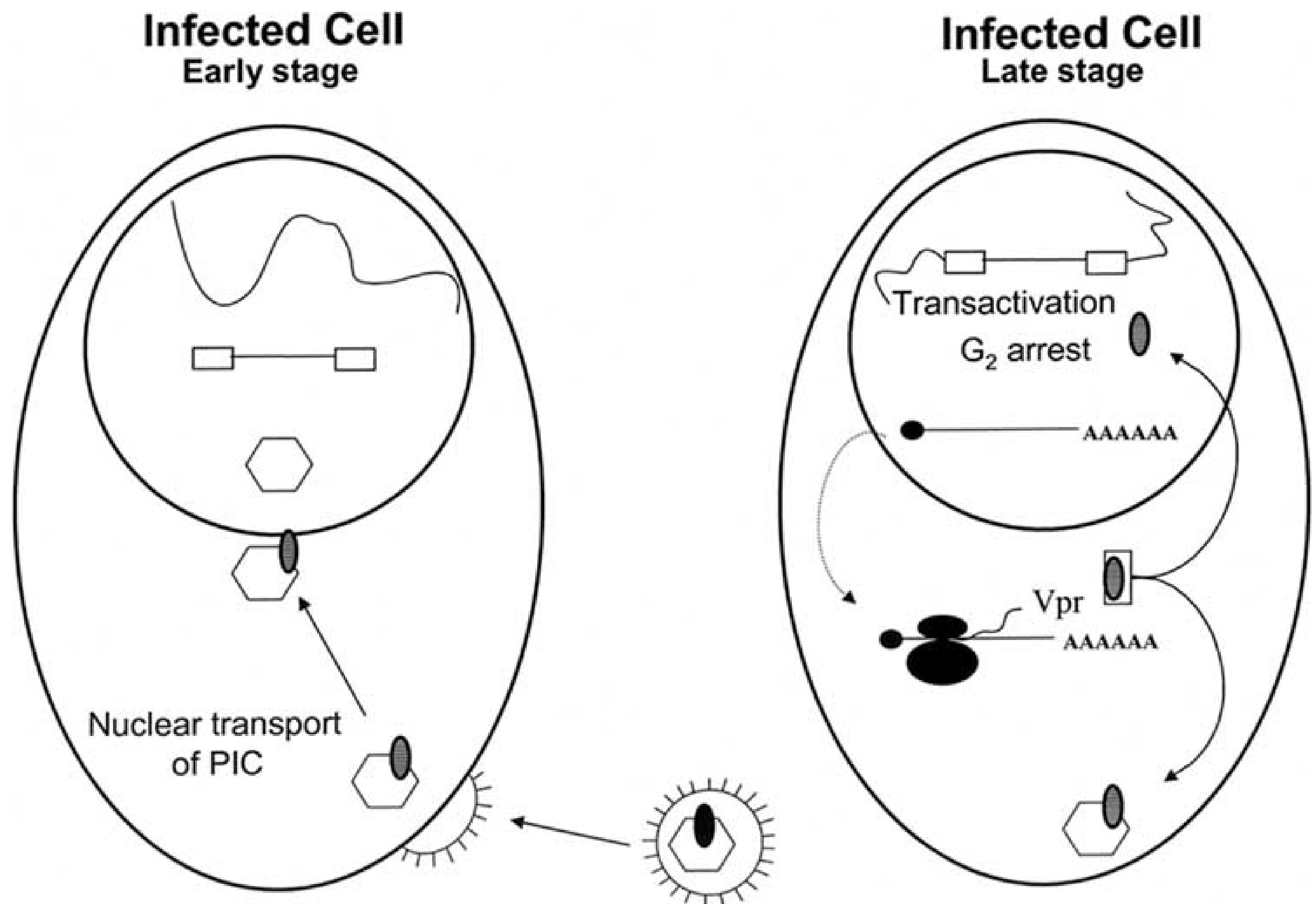

Fig. (1). Early and late functions of Vpr. In the early stage of an infection, Vpr, which exists in the virion in association with the viral capsid (hexagon) *via* an interaction with Gag p6, guides the pre-integration complex to the nucleus of an infected cell; this function is important in non-dividing cells, such as macrophages. Once integration has occurred, de novo-expressed Vpr induces cell cycle arrest in G_2 and consequent transactivation of the viral LTR; this function has been primarily characterized in dividing cells.

Interestingly, glucocorticoids also induce immunosuppressive signals, which has led to the speculation that the GR pathway may play a role in the immune deficiency observed in AIDS patients [59,83].

The GR, once bound to its glucocorticoid ligand, associates with steroid receptor coactivators (SRCs), such as the p300/CREB-binding protein (p300/CBP). The GR/SRC complex upregulates the expression of target genes *via* interactions between the GR complex and glucocorticoid responsive elements (GREs) within the promoter region of the target genes. GREs have been found within the HIV-1 LTR [40,57,75]. In addition, Refaeli *et al*. demonstrated that treatment of HIV-1 infected cells with mifepristone, an inhibitor of GR function, resulted in a 70% decrease in virus production [83]. Furthermore, the authors found that the defect in replication of viruses with mutations in *vpr*, could be compensated for by the addition of glucocorticosteroids in trans [83]. Vpr has been shown to upregulate transcription of cellular GRE-containing promoters *in vitro* [59,60,83,93], although it is currently unclear what role cellular GR-responsive genes may play in Vpr-induced G_2 arrest, and/or apoptosis.

It has been proposed that Vpr promotes transcription *via* the GR pathway by acting as an SRC [59,83,93]. One common feature among SRCs is the presence of the signature motif, LXXLL, which mediates SRC binding to the activation domain (AF-2) of the GR. Vpr possesses an LQQLL motif at amino acids 64-68 which, when mutated at multiple sites, renders Vpr defective in coactivation assays [93]. Furthermore, mutations within this motif abolished the ability of Vpr to bind the GR [93].

Recently, p300/CBP, has been implicated in regulation of HIV-1 transcription by Vpr [32,60]. In addition to its role in associating with the GR, p300/CBP also increases the DNA-binding affinity of nuclear factor-kappa B [36], a transcription factor implicated in regulation of the HIV-1 LTR [71]. Felzien *et al*. demonstrated that expression of Vpr led to activation of p300/CBP and a consequent increase in transcription of the HIV-1 LTR [32]. Kino *et al*. reported that Vpr, in addition to binding the GR, also binds p300/CBP [60]. The authors demonstrated that Vpr acts synergistically with overexpressed p300/CBP to drive transcription of HIV-1 LTR-luciferase reporter constructs, and increase viral replication *in vitro* [60].

Another protein, with a potential role in HIV-1 transcription, shown to interact with Vpr is the cellular transcription factor Sp1 [3,90,106]. The HIV-1 LTR contains three Sp1 binding motifs that function as positive

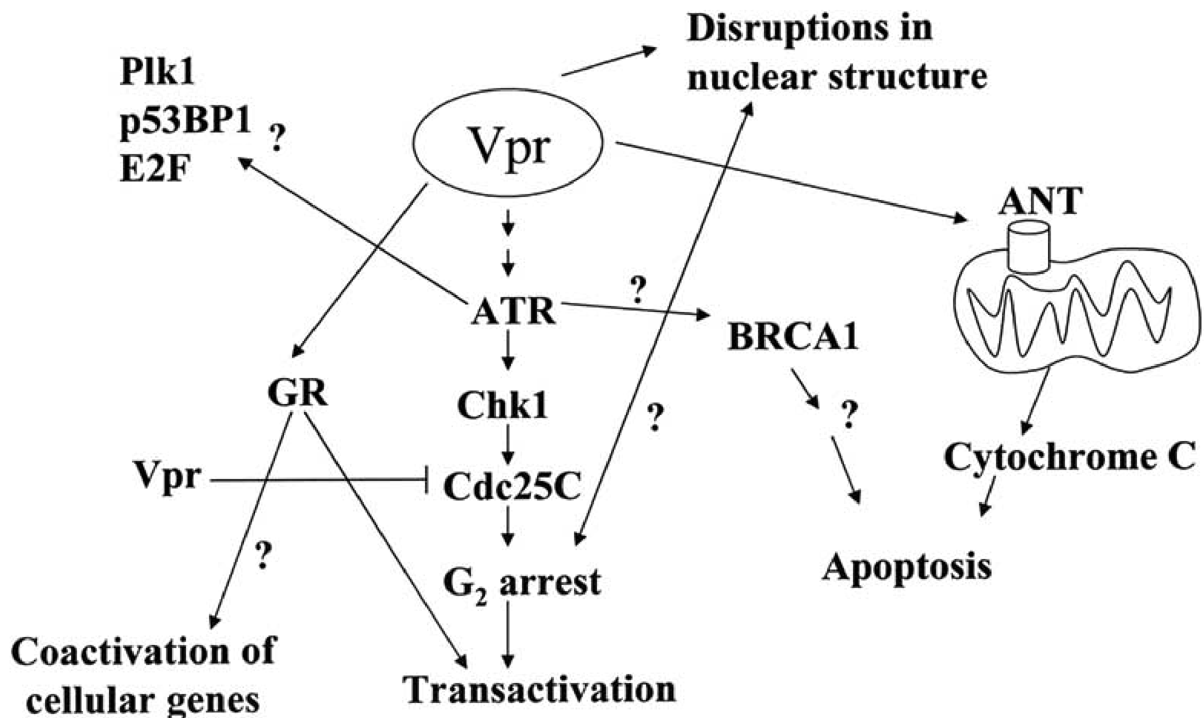

Fig. (2). Mecanism(s) underlying the late functions of Vpr. Various groups have suggested a variety of cellular pathways as being responsible for Vpr-induced cell cycle arrest, apoptosis, and transactivation of the viral LTR. Appropriate references are provided in the text.

regulatory elements. Wang *et al*. reported that Vpr interacts with Sp1 at the LTR Sp1 binding sites and cooperates with Sp1 in promoting LTR activity [106]. In addition, Sawaya *et al*. found that overexpression of *vpr* in cells with no endogenous Sp1 did not result in an upreguation of LTR activity, whereas overexpression of *vpr* in Sp1-expressing cells resulted in significant activation of the LTR [90].

In light of observations that Vpr-induced G_2 arrest correlates with transactivation of the HIV-1 LTR [42,87,117], it will be important to establish the relationship between G_2 arrest and other proposed mechanisms by which transcription of the LTR is upregulated. Currently, the potential role of G_2 arrest in promoting Sp1 activity is not known. In an attempt to examine the potential connection between G_2 arrest and p300/CBP activity, several studies have yielded conflicting results. Sherman *et al*. demonstrated that GR activation and induction of G_2 arrest were independent functions of Vpr, based on observations that Vpr mutants unable to induce G_2 arrest retained their ability to activate the GR pathway [93]. In addition, Kino *et al*. reported that Vpr mediates transactivation of the HIV-1 LTR by directly binding p300/CBP, which is independent of G_2 arrest [60]. In contrast, Felzien *et al*. observed that Vpr-induced p300/CBP activation and upregulation of the HIV-1 LTR were consequences of G_2 arrest [32].

NUCLEAR IMPORT OF PIC

The nuclear envelope is an insurmountable barrier to many retroviruses, which therefore require breakdown of the nuclear membrane during mitosis to gain access to the chromatin. One distinguishing feature of lentiviruses is their ability to induce active transport of PICs through the nuclear pore complex, thereby allowing these viruses to enter the nucleus and integrate into the genomes of non-dividing cells (reviewed in [17]) (Fig. (**1**)).

Three HIV-1 genes have been implicated in the infection of non-dividing cells: matrix [17], integrase [38], and Vpr [49], all of which contain nuclear localization signals. In addition, a cis-acting element within the *pol* gene termed the central DNA flap has been implicated in the nuclear import of proviral DNA [4,114]. Although Vpr is not required for *in vitro* viral replication in cell lines and activated peripheral blood lymphocytes (PBLs) [7,8,26,46,72], Vpr is required for efficient infection of non-dividing cells such as macrophages [49]. Specifically, capsid-associated Vpr plays a critical role in nuclear import of PICs [23,24,26,62,95,105] (Fig. (**1**)). In contrast with matrix and integrase, which both contain canonical NLSs, Vpr contains two unique NLSs that do not depend on RanGTP, transportin, or karyopherins for import into the nucleus [54,92]. Fouchier *et al*. demonstrated that Vpr interacts specifically with nucleoporin FG repeat regions [35]. This observation suggested a model in which Vpr interacts directly with the nuclear pore in order to facilitate nuclear import of PICs. Although the role of Vpr as a mediator of nuclear import in macrophages is undisputed, the precise mechanism by which Vpr induces nuclear transport of the PIC is still subject of debate.

CELL CYCLE DISRUPTION BY HIV-1 VPR

In 1995, several groups reported that Vpr is a cytostatic protein that specifically arrests cells in the G_2 phase of the cell cycle [48,55,82,85] (Fig. (**2**)). G_2 arrest is a general

response to DNA damage, in which the cell is given an opportunity to repair existing DNA damage prior to undergoing mitosis. Lack of effective DNA repair typically leads to self-destruction of the cell by apoptosis [73]. In the context of Vpr expression, cells arrested in G_2 ultimately undergo apoptosis [20,94,97,108,117]. Therefore, it is tempting to model studies on the mechanism of Vpr-induced G_2 arrest and apoptosis on existing knowledge about genotoxic insults in mammalian cells.

At first glance, it may seem counterintuitive that induction of cell cycle arrest and apoptosis would be advantageous to a retrovirus. The reasons for this idea are two-fold. First, under conditions of G_2 arrest, cellular protein translation is significantly down-regulated [14,81]. Second, induction of apoptosis would impede the budding of progeny virions through cellular membranes. Although the previous notions are correct, a model for HIV-1 infection of activated lymphocytes is emerging in which the virus establishes itself in the host cell, produces progeny virions in a burst during the G_2 phase of the cell cycle [42], and then actively induces apoptosis as a late event [117]. Recent findings suggest that cell cycle arrest in G_2 may actually serve the virus in at least two ways. First, the viral LTR is highly active in G_2 [42,52,117]. Therefore, G_2 arrest allows for more efficient viral replication *via* upregulation of viral transcription. Second, G_2 arrest may result in down-regulation of cap-dependent translation to favor protein translation from internal ribosome entry sites (IRESs), perhaps including a putative viral IRES [16]. Brasey *et al.* recently described the presence of an IRES element in the 5' leader of HIV-1 DNA [16]. The authors demonstrated that the HIV-1 IRES showed peak translation activity when cells were arrested in G_2.

Three protein kinases belonging to the phosphatidylinositol-3 kinase (PI3K) family are thought to be the major sensors of DNA damage in mammalian cells: the Ataxia Telangiectasia-mutated protein (ATM), the DNA-dependent protein kinase (DNA-PK), and the ATM- and Rad3-related protein (ATR) [12]. ATM and DNA-PK are primarily involved in sensing double-strand breaks while ATR is thought to play a key role in sensing stalled replication forks [27,28,116].

Bartz *et al.* investigated whether Vpr activated the well known sensors of DNA damage, p53 and ATM [10]. They found that Vpr-induced cell cycle arrest was independent of both p53 and ATM, and concluded that the mechanism of Vpr-induced arrest differed from canonical DNA damage response pathways known at the time [10]. Over the following years, the discovery of novel DNA damage sensors and a better understanding of the complex DNA damage signaling mechanisms has revealed new pathways in which Vpr may be acting to induce cell cycle arrest and apoptosis.

Our laboratory recently described ATR as being required for HIV-1 Vpr-induced G_2 arrest [87] (Fig. (**2**)). Using small interfering RNAs (siRNA) specific for ATR, or a dominant-negative ATR construct, Roshal and collaborators were able to effectively downregulate the levels of ATR protein and its function [87]. Under these conditions, Vpr-induced G_2 arrest was dramatically relieved and transactivation of the LTR was eliminated [87].

In response to stress signals, ATR can directly control eight known targets *via* serine/threonine phosphorylation [1,64,112,113]. These targets include the checkpoint kinase-1 (Chk1); Rad17; the breast cancer susceptibility protein 1 (BRCA1); the polo-like kinase-1 (PLK1); the histone 2A variant X (H2AX); the transcription factor, E2F; the tumor suppressor, p53; and the p53-binding protein-1 (53BP1). ATR targets, when activated, initiate signaling cascades that may result in three global effects: cell cycle blockade, recruitment of DNA repair and transcription factors, and induction of apoptosis (reviewed in [12]). Recently, we have examined the potential participation of three of the above ATR targets in the effects of Vpr [119]. Our studies addressed whether Rad17, a cell cycle regulator that forms a complex with ATR [121], was required for Vpr-induced G_2 arrest. We found that, as was previously shown for genotoxic agents like hydroxyurea [121], Rad17 elimination impaired activation of the G_2 checkpoint [119].

Activated ATR can also phosphorylate targets that are not required for activation of the G_2 checkpoint. One of these substrates is the histone 2A variant X (H2AX). H2AX can be found randomly distributed throughout chromatin, comprising approximately 10% of the total nucleosomal H2A [77]. H2AX has a highly conserved serine residue at position 139 that is absent in H2A, and can be phosphorylated by ATR and/or ATM [18,84,107]. Phosphorylated H2AX (γ-H2AX) is thought to amplify the DNA damage signal by enhancing and stabilizing recruitment of DNA damage sensor proteins such as ATR, ATM, Rad17, and the Rad9-Rad1-Hus1 complex, and DNA repair proteins such as BRCA1, Nbs1, Mre11 and Rad50 to sites of DNA damage [37]. We recently showed that H2AX is phosphorylated in the presence of Vpr, as evidenced by the formation of immunoreactive nuclear foci [119].

Activation of ATR often results in phosphorylation of another one of its targets: the breast cancer related protein-1 (BRCA1). BRCA1 is important for both checkpoint activation and DNA repair. BRCA1 co-localizes with DNA repair factors such as Rad51, PCNA, and Mre11-Rad50-Nbs1 [37]. It has been proposed that BRCA1 represents an essential link that coordinates cell cycle arrest with genomic repair and apoptosis (reviewed in [65]). Phosphorylation BRCA1 co-localizes to sites of DNA damage with γ-H2AX in the context of gentoxic insults. Activated BRCA1 also regulates the transcription of cellular genes such as Gadd45 (reviewed in [63]). We have recently shown that Vpr induces the formation of BRCA1 nuclear foci [119]. These findings suggest that Vpr-induced signaling through ATR may have cellular consequences other than G_2 arrest, such as initiation of DNA damage-like apoptotic signaling cascades (Andersen and Planelles, manuscript submitted), and/or recruitment of DNA repair complexes.

Much work lies ahead before we can achieve a thorough understanding of the mechanism by which Vpr triggers the ATR signaling pathway. Several possibilities could explain the activation of ATR by Vpr. First, Vpr may directly bind chromatin, mimicking DNA damage. Second, Vpr may activate ATR by a direct interaction with ATR or an ATR-associated protein. Third, Vpr may interfere with nuclear membrane-associated proteins thereby compromising nuclear membrane integrity and causing replication stress [30].

Other compelling models have been proposed to explain Vpr-induced G_2 arrest. Mahalingam *et al*, through yeast two-hybrid screening with Vpr as bait, found an interaction between Vpr and hVIP/MOV34, a member of the MOV34 protein family [67]. The MOV34 protein family includes members of the eukaryotic translation initiation factor 3 complex (eIF3), and several transcription factors that regulate cellular functions including cell division and cell fate determination [5,67,103]. Mahalingam *et al*. found that cells transfected with hVIP/MOV34 antisense RNA arrested in G_2 [67]. This finding suggests that hVIP/MOV34 may be a positive regulator of the G_2-to-M transition [67]. The authors inferred that Vpr could be preventing G_2 to M transition by interacting with, and inhibiting the function of hVIP/MOV34 [67]. While the precise connection between hVIP/MOV34 and cell cycle regulatory pathways is still unclear, the interaction between Vpr and hVIP/MOV34 remains an interesting model to explain Vpr-induced cell cycle arrest.

Withers-Ward *et al*. and Gragerov *et al*., using yeast two-hybrid screening, independently identified an interaction between Vpr and the human homologue of the yeast repair protein Rad23 (HHR23A) [43,110]. Co-transfection of HHR23A with Vpr resulted in a moderate but significant reduction in G_2 arrest [43,110]. This observation suggested that HHR23A, like hVIP/MOV34, may be a positive regulator of cell cycle progression. These data imply a model in which the interaction between Vpr and HHR23A somehow overcomes the effect of HHR23A and results in G_2 arrest. However, in contrast to previous findings, Mansky *et al*. reported that Vpr mutants unable to bind HHR23A still retained their G_2 arrest function [68]. In addition, Vpr from other primate lentiviruses, capable of inducing G_2 arrest, did not bind HHR23A *in vitro* [68].

The cyclin-dependent kinase inhibitor, $p21^{Waf1}$, was previously shown to be transcriptionally upregulated in a p53-dependent fashion, in the context of *vpr* expression [21]. This observation led the authors to formulate the hypothesis that $p21^{Waf1}$ may mediate Vpr-induced G_2 arrest, although this hypothesis was not tested [21]. A recent report demonstrates that transactivation of the *$p21^{Waf1}$* promoter by Vpr requires the presence of Sp1 binding sites and is p53-dependent [3]. Ectopic expression of Vpr also induces vigorous transactivation of the *$p21^{Waf1}$* promoter in bone marrow-derived CD34+ cells (Adam Tripp and Gerold Feuer, State U. of NY at Syracuse, personal communication). We recently reported that $p21^{Waf1}$-/- mouse embryonic fibroblasts are able to activate the G_2 checkpoint when transfected with Vpr [119]. This observation suggests that $p21^{Waf1}$ does not play a major role in mediating the G_2 arrest induced by Vpr in mouse cells, although our experiments cannot exclude that $p21^{Waf1}$ may have a minor contribution. Future experiments should address the potential alternative roles of $p21^{Waf1}$ upregulation by Vpr, including its ability to modulate apoptosis in certain settings (reviewed in [39]).

Goh *et al*. recently identified Cdc25C as an *in vivo* Vpr binding partner [41]. Cdc25C is a phosphatase that, when inactivated by Chk1 in response to DNA damage, fails to cleave an inhibitory phosphate at Tyr-15 of the Cdc2 kinase. Failure to dephosphorylate Cdc2 at Tyr-15 renders Cdc2 inactive and therefore unable to induce cell cycle progression beyond G_2. Goh *et al*. found that binding of Vpr to Cdc25C mapped near the catalytic domain of Cdc25C and inhibited Cdc25C phosphatase activity. The authors also observed that RNAi knockdown of Cdc25C abrogated Vpr-induced G_2 arrest (Fig. (**2**)). Of note, siRNA knockdown of either Cdc25C [41] or ATR [87] effectively abrogated Vpr-induced G_2 arrest. It is not known how binding of Vpr to Cdc25C could activate or involve ATR, considering that Cdc25C is thought to be downstream of ATR. Thus, whether both of the above observations are linked is unclear. It is possible that Vpr may be acting at two levels to inhibit G_2 to M transition, by binding Cdc25C and simultaneously activating the upstream steps of the ATR pathway.

De Noronha *et al*. observed, by time-lapse fluorescence microscopy, Vpr-induced disruptions in the nuclear envelope of Vpr-expressing cells (Fig. (**2**)). These disruptions were manifested as transient blebs protruding from the nuclear membrane into the cytoplasm. Importantly, Vpr mutants unable to induce G_2 arrest, failed to disrupt nuclear structure [30]. In addition, the authors did not observe similar alterations in nuclear structure in cells arrested in G_2 following treatment with the DNA-damaging agent, mitomycin C. This observation suggests that disruption of nuclear structure is unique to Vpr and may be the bridge linking Vpr to activation of the G_2 checkpoint. It is conceivable that disruptions in the nuclear envelope could lead to replication stress, thereby activating ATR and the G_2 checkpoint (Fig. (**2**)). Therefore, it will be interesting to examine whether Vpr is interacting with nuclear structural proteins directly or whether disruption of nuclear structure is a result of Vpr-induced G_2 arrest.

INDUCTION OF APOPTOSIS

The precise mechanism by which CD4+ T cells are lost over the course of an HIV-1 infection is poorly understood [47,88]. Several studies on HIV-1 induced cell death reported disparate observations. Finko *et al*. and Muro-Cacho *et al*., examining lymph nodes of HIV-1 infected patients, reported that cell death was predominantly occurring in uninfected “bystander” cells [33,69]. In contrast, Ho *et al*. and Wei *et al*. reported that HIV-1 replication leads to death of infected cells *in vivo* [50,109]. Several mechanisms have been proposed to explain the loss of CD4+ T cells in HIV-1-infected patients, including direct killing by HIV-1 replication, CD8+ T cell-mediated killing of infected CD4+ lymphocytes, and apoptosis of uninfected “bystander” cells. In addition, the HIV-1 proteins Tat, Rev, Vpu, Nef and Vpr have been implicated in the apoptosis of infected cells and bystander cells (reviewed in [88]). Considering that these mechanisms are not mutually exclusive, it is likely that any or all of them contribute to CD4+ T cell depletion over the course of HIV-1 infection.

Vpr has been reported to induce apoptosis following G_2 arrest in numerous cell types including primary T-cells, T-cell lines, and various other human transformed cell lines [6,79,80,94,97]. The mechanism by which Vpr induces apoptosis is not completely understood. In general, apoptosis can be triggered by extracellular signaling *via* death receptors (DRs) such as CD95 (Fas), by stress signals such as DNA damage, or, in the context of virus-infected cells, by CTL-mediated killing *via* release of cytotoxic

granules. The DR pathway is triggered by binding of the cognate ligand on the cell surface. Once engaged, the DR/ligand complex signals through intracellular adaptor proteins, which bind and activate caspase 8. Active caspase 8 triggers cleavage of Bid and activation of caspase 3, ultimately leading to apoptosis (reviewed in [102]).

Both the death receptors and DNA damage typically signal apoptosis *via* the mitochondria. In response to stress, a subfamily of proapoptotic Bcl-2-related proteins, namely Bid, Bim, Noxa and others, activate the Bax subfamily of Bcl-2 proteins [44]. Members of the Bax subfamily, which are normally loosely associated with the mitochondrial membrane or in the cytosol, oligomerize and become firmly established in the mitochondrial membrane, triggering the release of cytochrome C into the cytosol. Apaf-1 then binds to the released cytochrome C and this binding triggers the oligomerization of Apaf-1 and subsequent activation of caspase 9 (reviewed in [44]).

Muthumani *et al.* reported that Vpr-expressing cells underwent apoptosis *via* the mitochondrial pathway, characterized by cytochrome C release, and caspase 9 activation [70]. Interestingly, the authors did not observe activation of caspase 8, nor expression of Fas or its ligand in response to Vpr. This observation suggests that Vpr does not activate death receptor pathways and may induce apoptosis either through a stress-related pathway or *via* a direct interaction with the mitochondria.

Veira *et al.*, and Jacotot *et al.* have observed that, in a cell-free system, Vpr protein interacts with the intermembrane face of the adenine nucleotide transporter (ANT), within the mitochondrial membrane-associated permeability transition pore complex (PTPC), to cause ion permeability and swelling of fractionated mitochondria (Fig. (**2**)). *In vitro* binding of Vpr to ANT resulted in the release of cytochrome C [53,104]. These observations support a model in which Vpr induces mitochondrial depolarization directly rather than activating upstream stress signals. In contrast, Zhu *et al.* demonstrated that treatment of Vpr-infected cells with caffeine, an inhibitor of the DNA damage signaling proteins ATM and ATR, significantly reduced Vpr-induced apoptosis [117]. This observation indicates that Vpr first induces pro-apoptotic stress signals upstream of the mitochondria. In light of our recent data demonstrating a critical role for ATR in Vpr-induced G_2 arrest, we sought to examine whether RNAi-mediated knockdown of ATR could abrogate Vpr-induced apoptosis as it does G_2 arrest. We found that abrogation of Vpr-induced G_2 arrest with ATR-specific RNAi resulted in a dramatic decrease in apoptosis (Andersen and Planelles, manuscript submitted). These data are consistent with a model in which Vpr causes or mimics irreparable DNA damage and as a result, Vpr expressing cells arrest in G_2, and ultimately die by apoptosis.

The manner in which Vpr-induced cell cycle arrest and apoptosis may be linked remains a mystery. One possible link may be the ATR target, BRCA1 (Fig. (**2**)). Harkin *et al.* demonstrated that overexpressed BRCA1 transcriptionally upregulates Gadd45, which leads to JNK-dependent apoptosis [45]. Another potential link between G_2 arrest and apoptosis involves cellular translation from IRES elements active during G_2 arrest. Holcik *et al.* suggested that following a potentially deadly insult, such as DNA damage, IRES-dependent translation is upregulated, resulting in upregulation of both pro- and anti-apoptotic IRES-regulated genes such as Bag1, Apaf-1, XIAP, and cMyc (reviewed in [51]). In this model, cell fate is ultimately determined by the balance between pro- and anti-apoptotic proteins. Therefore, shortly after DNA damage, the cell may undergo a transient decision period, during which IRES-regulated pro- and anti-apoptotic proteins begin to accumulate. If the cell does not repair the damage within this decision period, pro-apoptotic proteins will tip the scale in favor of cell death.

IN VIVO VPR MUTATIONS: EFFECTS ON G_2 ARREST AND APOPTOSIS

Resistance to disease progression in LTNPs can be due to host factors such as innate host polymorphisms in HIV-1 receptors [25,120], or mutations within virus-encoded genes such as *nef* [13,31,58,61]. The Vpr mutation Q3R was identified in viruses isolated from an LTNP patient who demonstrated high levels of viremia, yet did not show significant loss of CD4+ lymphocytes [96]. Later, another Vpr mutation, R77Q, was found in 80% of virus isolates from a cohort of LTNPs [66]. The R77Q mutation was also present in a cohort of progressor patients, although with a lower frequency (33%) [66]. Interestingly, both the R77Q and Q3R mutants induce G_2 arrest, but induce apoptosis less efficiently, in comparison with wild type Vpr [66,96].

Jacotot *et al.* found that the minimal segment of Vpr able to bind ANT spans amino acids 72-83 [53]. This suggests that the decrease in apoptosis observed with Vpr R77Q may be a result of the inability of this Vpr mutant to effectively bind ANT and induce mitochondrial depolarization. The observation that knockdown of ATR or caffeine treatment reduce Vpr-induced apoptosis [87,117] points toward a model in which Vpr-induced apoptosis is directly related to induction of G_2 arrest. However, the observation that the R77Q and Q3R Vpr mutants induce wild-type levels of G_2 arrest but lower levels of apoptosis seems to support a model in which both effects are independent.

VPR IS CONSERVED THROUGH THE EVOLUTION OF PRIMATE LENTIVIRUSES

The sequence conservation of Vpr throughout the diversity of primate lentiviruses correlates with its functional conservation. The functions associated with HIV-1 Vpr have segregated in the HIV-2/SIVmac/SIVsm lineage. Both HIV-2 and SIVmac Vpr induce G_2 arrest but do not function to promote nuclear import of PICs [34,78]. In contrast, Vpx retains the ability to transport PICs to the nucleus but does not induce G_2 arrest [34,78].

In addition to the functions associated with HIV-1 Vpr, HIV-2 Vpx has evolved a novel function, namely binding to the MHC class II invariant chain (Ii) [74]. The cell surface presentation of exogenously-derived peptides by MHC class II molecules on the surfaces of antigen-presenting cells depends on the association between Ii and MHC class II within the ER and Golgi. Pancio *et al.* reported that cells stably expressing Vpx showed a marked decrease in Ii levels [74], which could, presumably, lead to a malfunction in

MHC class II antigen presentation. The authors proposed that the interaction between Vpx and Ii promoted enhanced Ii degradation.

SIVagm Vpr and HIV-1 Vpr share 31% amino acid identity and are functionally conserved in virion encapsidation, cell cycle arrest, and transactivation of the LTR [2,19,76,78,98,117]. However, interesting differences in the activities of these proteins have been observed. SIVagm Vpr mutants are unable to replicate in non-dividing cells, whereas HIV-1 Vpr mutants are able to replicate at low levels [19]. In contrast to HIV-1 *vpr*, the transactivation effect exerted by SIVagm Vpr is partly independent of its ability to induce G_2 arrest [117]. In addition, induction of apoptosis by SIVagm Vpr appears to be independent of G_2 arrest [117], which is in sharp contrast to the interdependence of G_2 arrest and apoptosis observed in the context of HIV-1 Vpr [117] (Andersen and Planelles, manuscript submitted).

CONCLUSION

HIV-1 Vpr has emerged as a critical pathogenic determinant of HIV-1, and it contributes to viral replication using multiple mechanisms. First, capsid-associated Vpr guides PICs to the nucleus to facilitate the infection of non-dividing cells. Second, Vpr induces G_2 arrest to promote transcription from the viral LTR. In addition, Vpr-mediated activation of p300/CBP and GR pathways may influence transcription of cellular genes and activate transcription from the viral LTR. Third, induction of G_2 arrest by Vpr may upregulate protein translation from an IRES element within the HIV-1 5' leader, thereby promoting viral replication at the level of translation. In addition, induction of apoptosis by Vpr may play a role in depletion of CD4+ lymphocytes *in vivo*. In support of this idea, the discovery of Vpr mutations in LTNPs that render Vpr less cytotoxic *in vitro*, underscore a role for Vpr in CD4+ T cell depletion in HIV-1-infected patients. Detailed information regarding the pathways used by Vpr to induce cell cycle arrest, upregulate viral transcription, and induce apoptosis, may lead to novel anti-HIV-1 therapies based on blocking these functions.

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