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HIV-1 Vpr: Mechanisms of G2 Arrest and Apoptosis

Joshua L. Andersen¹, Erwann Le Rouzic^{2,3}, and Vicente Planelles⁴

¹Center for the Study of Aging and Human Development, Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710

²Institut Cochin, Université Paris Descartes, CNRS (UMR 8104), Paris, France

³Inserm, U567, Paris, France

⁴Division of Cellular Biology and Immunology, Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84132

Abstract

Since the first isolation of HIV-1 from a patient with generalized lymphadenopathy in 1983, great progress has been made in understanding the viral life cycle and the functional nuances of each of the nine genes encoded by HIV-1. Considerable attention has been paid to four small HIV-1 open reading frames, *vif*, *vpr*, *vpu* and *nef*. These genes were originally termed “accessory” because their deletion failed to completely disable viral replication *in vitro*. More than twenty years after the cloning and sequencing of HIV-1, a great deal of information is available regarding the multiple functions of the accessory proteins and it is well accepted that, collectively, these gene products modulate the host cell biology to favor viral replication, and that they are largely responsible for the pathogenesis of HIV-1. Expression of Vpr, in particular, leads to cell cycle arrest in G₂, followed by apoptosis. Here we summarize our current understanding of Vpr biology with a focus on Vpr-induced G₂ arrest and apoptosis.

Introduction

HIV-1 Vpr

The first clue as to the role of Vpr in the viral life cycle came from the observation that truncation of the open reading frame resulted in a slower-replicating virus (Hattori et al., 1990; Ogawa et al., 1989; Wong-Staal et al., 1987) and hence its name, viral protein, regulatory. HIV-1 Vpr is a small 96 amino acid (14 kDa) protein that is delivered in two modes during infection. *De novo* expressed Vpr appears during the late phase of infection (Schwartz et al., 1991), while Vpr protein is also packaged into nascent virus particles via an interaction with Gag p6 (reviewed in (Tungturthi et al., 2003)).

The precise contribution of Vpr to HIV-1 pathogenesis *in vivo* is difficult to determine, in part because Vpr is highly conserved. However, SIV studies from primate models suggest that the functions of Vpr are critical to AIDS progression. In the HIV-2/SIVmac/SIVsm lineage, the functions associated with HIV-1 Vpr have segregated into two genes, *vpr* and *vpx* (Tristem et

*Corresponding author: Vicente Planelles, Ph.D., Department of Pathology, University of Utah School of Medicine, 15 North Medical Drive East #2100, Salt Lake City, UT 84132, Phone (801)581-8655, email: vicente.planelles@path.utah.edu.

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al., 1992). While deletion of either SIVmac *vpr* or *vpx* alone only modestly reduces *in vivo* viral replication, deletion of both genes generates a severely crippled virus incapable of causing disease (Gibbs et al., 1995; Lang et al., 1993).

In vitro analysis has illustrated various Vpr functions that could potentially contribute to HIV-1 pathogenesis *in vivo*. These functions are transactivation of the HIV-1 long terminal repeat (LTR), nuclear import of preintegration complexes (PICs), induction of cell cycle arrest in G₂, and apoptosis in infected cells.

Structure of HIV-1 Vpr

Several approaches to model the structure of Vpr by NMR and circular dichroism have been used with success (Henklein et al., 2000; Schuler et al., 1999; Wecker et al., 2002; Wecker and Roques, 1999). The first view of Vpr structure came from studies in which the N and C terminal halves of Vpr were analyzed separately by NMR (Schuler et al., 1999; Wecker and Roques, 1999). As had been predicted, amino acids 1–51 of Vpr form a α helix turn- α helix structure, while amino acids 52–96 form another α helix rich in leucine residues that has been proposed to form a leucine zipper-like structure (Bourbigot et al., 2004; Schuler et al., 1999; Wecker and Roques, 1999). Analysis of full-length Vpr in semi-hydrophobic solvent (CD₃CN), confirmed earlier predictions showing the presence of the three amphipathic α helices folded around a hydrophobic core (Morellet et al., 2003). These helices are flanked by a flexible, negatively charged N-terminal domain, and a flexible, positively charged C-terminal domain rich in arginine residues (Morellet et al., 2003). Specific C-terminal arginines, such as R80, have been proposed to play a role in Vpr signaling and protein/protein interactions leading to G₂ arrest and apoptosis (Gaynor and Chen, 2001; Lum et al., 2003).

Vpr sequence variation

The *vpr* gene is highly conserved *in vivo*, not only in HIV-1 isolates from different geographical origins, but also through the evolution of primate lentiviruses (Fletcher et al., 1996; Planelles et al., 1996; Tristem et al., 1998). However, due to the limited genetic variation of *vpr in vivo*, a direct link with viral pathogenesis remains largely theoretical. Two studies have provided compelling evidence that there is strong selection in favor of the *vpr* gene *in vivo*. In a vaccine study conducted in chimpanzees (Fultz et al., 1992), two animals were challenged with a virus stock derived from HIV_{IIIB}, which encodes a truncated, unstable and non-functional Vpr protein. Goh and colleagues (Goh et al., 1998) performed a retrospective, longitudinal analysis of the *vpr* gene sequence in this animals virus populations, which revealed restoration of the truncated open reading frame in both chimpanzees at 6 to 8 weeks and at 2 years post-inoculation, respectively (Goh et al., 1998). The second study resulted from an unfortunate accident in which a laboratory worker became infected with a stock of HIV_{IIIB}, which also contained the above inactivating mutation in *vpr* (Reitz et al., 1994; Weiss et al., 1988). Sequence analysis of virus from peripheral blood cells from this individual two years after infection revealed that the *vpr* gene reverted to full-length (Goh et al., 1998). Thus, there is positive selection for Vpr function *in vivo*.

More recently, various surveys of *vpr* sequences in long-term non-progressors (LTNP) populations have found non-synonymous nucleotide substitutions in *vpr*. 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 (Somasundaran et al., 2002). Later, another Vpr mutation, R77Q, was found in 80% of virus isolates from a cohort of LTNPs (Lum et al., 2003). The R77Q mutation was also present in a cohort of progressor patients, although with a lower frequency (33%) (Lum et al., 2003). Interestingly, both the R77Q and Q3R mutants induce G₂ arrest, but induce apoptosis less efficiently, in comparison with wild-type Vpr (Lum et al., 2003; Somasundaran et al., 2002). In both studies, it was tempting to

speculate that the reduction in Vpr-associated cytotoxicity might account for the nonprogressive clinical course in patients (Lum et al., 2003; Somasundaran et al., 2002). However, the association of R77Q with a non-progressive clinical phenotype has recently been questioned in two separate studies (Fischer et al., 2004; Rodes et al., 2004). Both studies sequenced virus from cohorts of LTNP and compared these sequences with those from a progressor cohort, and observed that the R77Q mutation was found in both progressor and non-progressor cohorts at statistically equivalent frequencies (Fischer et al., 2004; Rodes et al., 2004). A more recent analysis of the GenBank database revealed that R77 predominates in subtype B, whereas Q77 predominates in clades A, C, D, G, H and groups O and N viruses (Rajan et al., 2006). In contrast, subtype F and K strains often contain H77 (Rajan et al., 2006). Rajan et al. also observed that a virus carrying Vpr Q77 induced less cytopathicity if it had R5 tropism, but not if it had X4 tropism (Rajan et al., 2006).

A recent study has analyzed the impact of the Vpr(R77Q) (Andersen et al., 2006) and Vpr (Q3R) (JA and VP, unpublished results) polymorphisms on Vpr function *in vitro*. The levels of induction of both G₂ arrest and apoptosis were compared between wild-type Vpr and Vpr (R77Q) or Vpr(Q3R) and found that expression of either mutant resulted in normal, and not lower, levels of apoptosis induction. Two other surveys of viral sequences in LTNP populations found *vpr* to be highly conserved, and did not identify amino acid substitutions predicted to impact Vpr function (Alexander et al., 2000; Zhang et al., 1997).

Vpr from other primate lentiviruses

Vpr is conserved in five of the primate lentiviral lineages, including HIV-1/SIVcpz, HIV-2/SIVmac/SIVsm, SIVagm, SIVsyk, and SIVmnd (Tristem et al., 1998). SIV isolates from other primates including red-capped mangabey, mona, and mustached have been found to express Vpr although it is unclear whether Vpr from these SIV strains is functionally analogous to HIV-1 Vpr (Barlow et al., 2003; Beer et al., 2001; Courgnaud et al., 2003; Takemura and Hayami, 2004).

An interesting exception to the conservation of Vpr in the primate lentiviral lineages is the HIV-2/SIVmac/SIVsm lineage, in which the functions of Vpr have segregated into two genes, termed Vpr, and Vpx. Tristem et al. proposed that Vpx arose as a result of homologous recombination between SIVagm and an ancestor of HIV-2 (Tristem et al., 1992; Tristem et al., 1998). Both HIV-2 and SIVmac Vpr induce G₂ arrest, but unlike Vpr from other lineages, these Vpr proteins do not assist in PIC nuclear import, a role taken over by Vpx (Fletcher et al., 1996; Planelles et al., 1996). Moreover, HIV-2 Vpx appears to exert a novel function by binding to the MHC class II invariant chain (Ii) and causing Ii degradation (Pancio et al., 2000). 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 (Pancio et al., 2000), which could lead to a malfunction in MHC class II antigen presentation.

Other studies on interspecies differences in Vpr have focused on SIVagm Vpr and HIV-1 Vpr, which share 31% amino acid identity and are functionally conserved in virion encapsidation, cell cycle arrest, and transactivation of the LTR (Accola et al., 1999; Campbell and Hirsch, 1997; Philippon et al., 1999; Planelles et al., 1996; Stivahtis et al., 1997; Zhu et al., 2001). However, some differences between SIVagm Vpr and HIV-1 Vpr have been observed. One marked difference is that while HIV-1 lacking Vpr is able to replicate *in vitro*, Vpr-null SIVagm is severely crippled and fails to replicate *in vitro* (Campbell and Hirsch, 1997). In addition, LTR transactivation and apoptosis induced by SIVagm Vpr appear to be at least partially independent of G₂ arrest, in contrast with the interdependence that occurs in the context of HIV-1 Vpr (Zhu et al., 2001).

G₂ Cell Cycle Arrest and Vpr

G₂ arrest and DNA damage

Cell cycle arrest in G₂ has been characterized in detail in the context of DNA damage so it is in this field that we understand the molecular pathways leading to, and the cellular consequences of, cell cycle checkpoint activation. Cells have evolved a variety of response pathways to protect the integrity of their genomes and have coordinated these pathways with cell cycle progression and apoptosis. The objectives of these pathways are to excise damaged DNA, rejoin DNA strand breaks, or directly reverse lesions with the ultimate goal of preventing propagation of DNA mutations in S phase or through cell division. Accordingly, checkpoint activation leads to cell cycle arrest prior to DNA replication (G₁/S arrest) or prior to mitosis (G₂/M arrest) to allow time for repair. If the damage is irreparable, the cell dies by apoptosis.

ATM and ATR: Regulators of the G₂/M checkpoint in response to genotoxic stress

The cascade of events leading to cell cycle arrest is initiated when sentinel proteins within the nucleus recognize alterations in DNA/chromatin structure. These early-responding proteins include ataxia telangiectasia-mutated and Rad3-related (ATR), and ataxia telangiectasia-mutated (ATM). ATR and ATM can be categorized based on the type of lesion they respond to although some overlap in function does occur. In general, ATM is primarily activated by double strand breaks (DSBs), while ATR responds to a wider range lesions, but most notably, stalled replication forks (reviewed in (Harrison and Haber, 2006)).

The gene that encodes ATM is mutated in the autosomal recessive disorder, ataxia telangiectasia (A-T). A-T patients suffer from chromosomal instability, cancer susceptibility, growth retardation, cerebellar degeneration and immunodeficiency. In the absence of DNA damage, ATM exists as an inactive dimer with the kinase domain of each molecule concealed within its homodimeric binding region. Exposure of cells to ionizing radiation leads to ATM autophosphorylation at residue 1981 and dissociation of the dimer, which exposes the kinase domain and thus allows ATM to phosphorylate substrates (reviewed in (Ward and Chen, 2004)). Recruitment of ATM to sites of damage is facilitated by a group of cofactors including Mre11, Rad50, and Nbs1 (Bakkenist and Kastan, 2003; Falck et al., 2005).

The first identified target of ATM phosphorylation was Ser-15 of p53, a residue important for p53 activation (Siliciano et al., 1997). Once activated, p53 triggers upregulation of several target genes including p21^{waf1/CIP1}, which inactivates cyclin E/Cdk2 complexes and prevents G₁-to-S-phase progression (reviewed in (Agarwal et al., 1998)). Other targets of ATM include Chk2 (Thr-68), and BRCA1 (Ser-1423), which are involved in activation of cell cycle checkpoints, apoptosis and DNA repair (Cortez et al., 1999; Matsuoka et al., 1998).

In contrast to ATM, early studies in mammalian cell systems proposed a model in which the kinase activity of ATR remains constant regardless of the presence or absence of damage (Falck et al., 2005; Zou and Elledge, 2003). The ATR model, based on these studies, predicts that the kinase properties of ATR are focused as ATR protein is recruited to sites of DNA damage via an interaction with ATR-interacting protein (ATRIP)/replication protein A (RPA)-coated single stranded DNA (ssDNA). One significant extension of this model is that it predicts a mechanism by which the ATR-dependent checkpoint is activated: the aberrant persistence of RPA at sites of stalled replication (as opposed to transient RPA coating of ssDNA at sites of ongoing replication) triggers ATR checkpoint activation. However, this model has been challenged by recent work in *Xenopus* in which depletion of RPA did not abrogate the ability of ATR to respond to replication stress (Kumagai et al., 2006). Rather, topoisomerase II β binding protein 1 (TopBP1) was found to directly modulate the activity of ATR in response to replication stress (Kumagai et al., 2006). These data suggest an alternative model in which

ATR kinase activity can be modulated by TopBP1 independent of the association between ATR/ATRIP and RPA.

Activated ATR phosphorylates a number of targets implicated in control of the G₂ checkpoint including H2AX, Rad17, BRCA1, p53, and Chk1 (Bao et al., 2001; Chen, 2000; Foray et al., 2003; Guo et al., 2000; Tibbetts et al., 1999; Tibbetts et al., 2000; Ward and Chen, 2001; Zou et al., 2002). Phosphorylation of these targets by ATR can have wide-ranging effects (Figure 1). For instance, phosphorylation of BRCA1 initiates the upregulation of genes involved in DNA repair, cell cycle arrest, apoptosis, and gene expression (reviewed in (Lane, 2004)), such as Gadd45 α (Jin et al., 2000). In addition, phosphorylation of Chk1 leads to phosphorylation/inactivation of Cdc25c, which in turn allows for activation of Wee1 and consequent inactivation of Cdk1, resulting in G₂ arrest (reviewed in (Zhou and Elledge, 2000)).

Vpr-induced G₂ arrest and viral replication

Several reports in 1995 described Vpr as a cytostatic protein that arrests cells in the G₂ phase of the cell cycle (He et al., 1995; Jowett et al., 1995; Re et al., 1995; Rogel et al., 1995). The *in vivo* significance of Vpr-induced G₂ arrest has been difficult to determine, but *in vitro* studies suggest that the viral promoter, or long terminal repeat (LTR), is highly active in the G₂ phase of the cell cycle (Goh et al., 1998; Hrimech et al., 1999; Zhu et al., 2001). Therefore, G₂ arrest may promote optimal transcription from the LTR, which in turn promotes an increase in viral output. One study found that the decrease in LTR transactivation observed with Vpr-deficient virus could be recovered by the addition of pharmacological inducers of G₂ arrest, which supports the notion that G₂ arrest alone, and not some other property of Vpr, is sufficient for LTR transactivation (Goh et al., 1998). In addition, G₂ arrest fosters a cellular environment in which cap-dependent translation is diminished in favor of translation from internal ribosome entry sites (IRESs), common to many viruses including the well-characterized picornaviruses and cricket paralysis viruses (reviewed in (Gallego, 2002)). One study from Brasey et al. described the presence of an IRES element in the 5' leader of HIV-1, which showed peak translational activity in G₂ (Brasey et al., 2003). Thus, G₂ arrest induced by Vpr may promote virus production by upregulating both transcription (via the LTR) and translation (via the putative HIV-1 IRES) of viral products. Recently, Sakai et al. reported that deletion of Vpr, in addition to Vif, rendered HIV-1 incapable of causing G₂ arrest and eliminated the cytopathic properties of the virus, which suggests a link between viral cytopathicity and cell cycle arrest (Sakai et al., 2006).

Recently, infected lymphocytes (identified by expression of Gag p24) isolated from recent HIV-1 seroconverters and stained with propidium iodide for DNA content were found to be arrested in G₂, indicating that G₂ arrest is not merely an *in vitro* phenomenon (Zimmerman et al., 2006).

Mechanisms to explain Vpr-induced G₂ arrest

Early work on Vpr-induced G₂ arrest found that the effect of Vpr on cells was similar to the effects of the alkylating agent, nitrogen mustard, in that both caused a decrease in Cdk1 kinase activity leading to a cell cycle arrest that could be partially reversed by methylxanthines (Poon et al., 1997). In another early study it was found that Vpr-induced cell cycle arrest was not dependent on ATM or p53, and concluded that the mechanism of Vpr-induced arrest differed from canonical DNA damage response pathways (Bartz et al., 1996). While this conclusion may have been appropriate based on the understanding of DNA damage pathways at the time, over the following years the emergence of novel DNA damage-sensing proteins has revealed new pathways on which Vpr acts to induce cell cycle arrest.

Our laboratory described ATR (Figure 1) as being required for HIV-1 Vpr-induced G₂ arrest (Roshal et al., 2003). Using small interfering RNAs (siRNA) specific for ATR, or a dominant-negative ATR construct, Roshal et al. were able to effectively downregulate the levels of ATR protein or its function (Roshal et al., 2003). Under these conditions, Vpr-induced G₂ arrest was relieved and transactivation of the LTR was eliminated (Roshal et al., 2003). In a subsequent report, it was shown that downstream components of the ATR pathway are activated by Vpr, including the phosphorylated histone 2A variant-X (γ -H2AX), and BRCA1 (Andersen et al., 2005; Zimmerman et al., 2004).

Recently, Zimmerman et al. reported that HIV-1 infection in primary human CD4⁺ lymphocytes results in ATR-dependent G₂ arrest and accumulation of RPA, which suggests the presence of stalled replication forks (Zimmerman et al., 2006). Consistent with a model in which Vpr activates the ATR DNA damage response pathway, Yuan et al. found that Vpr activates the negative regulator of Cdk1, Wee1 (Yuan et al., 2003). They further showed that Vpr is unable to induce G₂ arrest in cells in which Wee1 has been depleted by siRNA (Yuan et al., 2003). In addition, Lai et al. demonstrated that Vpr causes foci formation of the ATR-associated molecules 53BP1 and RPA, in addition to Chk1 phosphorylation at a residue specifically phosphorylated by ATR (Lai et al., 2005). Interestingly, the authors also demonstrated that a GFP-tagged Vpr protein could be observed binding to chromatin in intact cells, while C-terminal mutants of Vpr, defective in causing G₂ arrest, were unable to bind chromatin, suggesting that these two functions of Vpr may be linked (Lai et al., 2005). While these data suggest that Vpr may directly cause damage to DNA, analysis of DNA damage by pulse field gel electrophoresis (PFGE) failed to reveal the presence of DSBs by Vpr (Lai et al., 2005). These data were in conflict by results obtained by Tachiwana et al., who detected Vpr-induced DSBs by PFGE (Tachiwana et al., 2006), and it is unclear, given the similar experimental systems used by these groups, how the apparent contradiction can be explained. However, the dispensability of ATM in Vpr-induced G₂ arrest (Bartz et al., 1996; Zimmerman et al., 2004), and the lack of ATM activation/phosphorylation in Vpr-expressing G₂-arrested cells (Lai et al., 2005) strongly support a model in which Vpr activates the checkpoint by a mechanism that does not involve DSBs. In addition, the presence of RPA foci and the strict requirement for ATR and its downstream targets (Lai et al., 2005; Zimmerman et al., 2006), raises the possibility that Vpr, either directly through interaction with chromatin or replication machinery, or indirectly via interference with nuclear structure or the proteasome pathway, causes replication stress.

A possible clue to explain Vpr-induced G₂ arrest was provided by studies by de Noronha et al. in which they captured, by live-cell microscopy, the presence of dynamic herniations of the nuclear envelope, concomitant with changes in the subcellular distribution of Cyclin B1, Wee1, and Cdc25C in Vpr-expressing cells (de Noronha et al., 2001). Nuclear herniations were observed as the Vpr-expressing cells transitioned through S phase. These herniations were found to contain DNA, and defects in lamin structure were observed around the nuclear lesions. However de Noronha et al. were unable to detect a direct interaction between Vpr and nuclear lamins, nor an increase in Vpr protein level around or within the nuclear herniations. Importantly, nuclear herniations were not observed in cells expressing Vpr mutants defective in inducing G₂ arrest. Additionally, nuclear herniations were not observed in cells treated with pharmacological inducers of G₂ arrest and apoptosis, suggesting a difference in the mechanism by which Vpr induces G₂ arrest compared to genotoxic agents (de Noronha et al., 2001).

What could be the link between disruptions in nuclear architecture and G₂ arrest by Vpr? Nuclear herniations would likely correlate with or cause alterations in chromatin structure and therefore affect the processivity of DNA replication. Indeed, disruption of nuclear lamin structure impacts the DNA replication checkpoint response (Liu et al., 2006; Manju et al., 2006). A model in which Vpr causes replication stress by inducing herniations would be

consistent with the body of literature demonstrating that Vpr specifically activates ATR pathway. However, it remains to be seen whether the nuclear herniations are the Vpr-induced stimulus to activate ATR or, alternatively, a downstream result of Vpr-induced ATR activation.

It is noteworthy that Vpr possesses a great affinity for the nuclear envelope and several groups reported that Vpr specifically interacts with components of the nuclear pore complex (Fouchier et al., 1998; Le Rouzic et al., 2002; Popov et al., 1998; Vodicka et al., 1998). The possibility that Vpr directly acts at this level to perturb the nuclear architecture and consequently induces G₂ arrest remains open. It remains to be seen whether Vpr mutants can be identified which dissect Vpr's cyostatic activity from its localization at the nuclear envelope.

Vpr binding partners involved in G₂ arrest

In vitro mutational analysis of Vpr has shown that the determinants of G₂ arrest are primarily located in the C-terminal region of Vpr although the biological significance of this region, in terms of binding partners or signaling, has not been elucidated. Vpr has been shown to bind to numerous targets *in vitro*, with several implicated in the induction of G₂ arrest. However, no consensus as to the biologically relevant Vpr-binding protein has been met until recently. In the following paragraphs, we will summarize many of the arguments surrounding the putative Vpr binding proteins and conclude by summarizing recent data from several groups that demonstrate a role for Vpr in modulating the activity of Cullin RING ubiquitin ligases toward induction of G₂ arrest.

Vpr was found in complex with the glucocorticoid receptor (GR). A third protein found in the Vpr/GR complex, termed Vpr-interacting protein (VIP), was identified through a yeast two-hybrid approach and was proposed to be associated with G₂ arrest induction by Vpr (Mahalingam et al., 1998). Although the physiological role of VIP remains undetermined, it was proposed to be a member of the eukaryotic initiation factor 3 (eIF3) complex, which regulates transcription and is essential for G₁/S and G₂/M progression. However, Sherman et al. found that G₂ arrest and GR-binding are independent phenotypes of Vpr based on experiments in which Vpr mutants defective for G₂ arrest retained their ability to bind the GR (Sherman et al., 2000). A recent report by Muthumani et al. demonstrated that the Vpr/GR interaction prevents PARP-1 accumulation in the nucleus and therefore may primarily affect gene transcription by interfering with NF- κ B (Muthumani et al., 2006).

Vpr was shown to bind uracil DNA glycosylase (UNG) and the human homologue of rad 23 A (HHR23A) (Gragerov et al., 1998; Withers-Ward et al., 1997). Both of these proteins are involved in the DNA repair process so they were naturally suspected of playing a role in Vpr-induced G₂ arrest. However, subsequent analysis showed that there is no correlation between the association of HHR23A or UNG with Vpr and the induction of G₂ arrest (Kaiser and Emerman, 2006; Mansky et al., 2001). In addition, Goh et al. reported that Vpr binds and inactivates the positive regulator of Cdk1, Cdc25, and mutation of the Cdc25-binding region within Vpr rendered Vpr deficient in G₂ arrest induction (Goh et al., 2004). However, the observation that Vpr activates molecules upstream of Cdc25, such as ATR and Chk1, suggests that inactivation of Cdc25 may be a consequence of Vpr-mediated activation of upstream kinases (specifically, Chk1) rather than a direct effect of Vpr.

Vpr has also been shown to bind Cyclophilin A (CypA), a peptidyl prolyl *cis-trans* isomerase, involved in maintenance of protein conformation by *cis-trans* interconversion of N-terminal peptide bonds. Zander et al. reported that CypA is required for the stability and function of Vpr (Zander et al., 2003). They further showed that Vpr mutated at proline 35 is unable to bind CypA nor induce G₂ arrest (Zander et al., 2003). In an effort to investigate the significance of the Vpr-CypA interaction, our group demonstrated that abolishment of the interaction (either by using CypA-deficient cells, incubating with Cyclosporin A, or by mutating Vpr at proline

35) does not abrogate Vpr-induced G₂ arrest (Ardon et al., 2006). Therefore, binding to CypA and induction of G₂ arrest are independent activities of Vpr.

Another proposed Vpr binding partner is Hsp70, an abundant stress-regulated cellular protein (Bukrinsky and Zhao, 2004; Iordanskiy et al., 2004a; Iordanskiy et al., 2004b). Bukrinsky and colleagues reported that, in contrast to other Vpr-binding molecules, Hsp70 interacted with Vpr to inhibit, rather than induce, G₂ arrest (Bukrinsky and Zhao, 2004). The authors proposed that Hsp70 is induced by HIV-1 infection as part of an innate antiviral response to oppose the pathogenic properties of Vpr. The authors also showed that Hsp70 inhibits replication of Vpr-proficient HIV-1, but not Vpr-deficient HIV-1 (Iordanskiy et al., 2004a; Iordanskiy et al., 2004b).

A recent report demonstrated that Vpr interacts with SAP145, a subunit of Sf3b required for the splicing of precursor mRNAs (Terada and Yasuda, 2006). In their study, Terada and Yasuda found that full-length Vpr, but not Vpr lacking the C terminus, bound SAP145 and that Vpr-SAP145 binding prevented an association between SAP145 and SAP49 (Terada and Yasuda, 2006). The authors also found that depletion of either SAP145 or SAP49 caused G₂ arrest and the induction of γ H2AX and BRCA1 nuclear foci formation (Terada and Yasuda, 2006), as has been observed in the context of expression of Vpr alone (Zimmerman et al., 2004). It is unclear how the Vpr/SAP145 interaction may lead to G₂ arrest, as inhibition of SAP145 did not alter the splicing of genes required for cell cycle progression such as Cyclin B1 or Cdk1 (Terada and Yasuda, 2006). In a subsequent report, Aida and colleagues corroborated the Vpr/SAP145 interaction and found that SAP145 is required for Vpr-induced inhibition of processing of certain pre-mRNAs such as immunoglobulin M, and β -globin (Hashizume et al., 2007). However, the authors found that Vpr(R80A), a Vpr mutant defective for G₂ arrest, could bind SAP145 and inhibit pre-mRNA processing (Hashizume et al., 2007), suggesting that the Vpr/SAP145 interaction *per se* is not sufficient to induce G₂ arrest.

In 1993, Scheffner and colleagues published a seminal paper in which they demonstrated that E6 proteins from human papilloma virus (HPV) types 16 and 18 caused polyubiquitination and degradation of the tumor suppressor p53 (Scheffner et al., 1993). By causing degradation of p53, HPV is able to suppress cell cycle arrest and apoptosis of the infected cell, and thereby replicate under more favorable cellular conditions. This discovery was the first of several describing the ability of viral proteins to modulate the ubiquitin proteasome system (UPS). The list of viral proteins that can manipulate the UPS now includes the paramyxovirus V protein, the hepatitis B virus protein X, and the HIV-1 proteins, Vpu and Vif (Horvath, 2004; Leupin et al., 2005; Margottin et al., 1998; Mehle et al., 2004; Sheehy et al., 2003; Sitterlin et al., 2000; Yu et al., 2003), and as its most recent addition, HIV-1 Vpr (reviewed in (Dehart and Planelles, 2007))

After years of searching for a mechanism to explain Vpr-induced G₂ arrest, it is somewhat perplexing that the most informative Vpr-binding partner was discovered in 1994 when a protein of unknown function was co-precipitated with Vpr (Zhao et al., 1994). This protein was originally named Vpr-interacting protein (RIP) or Vpr binding protein (VprBP; see Figure 1), and its cellular function remained enigmatic until 2006 when several groups identified a family of proteins, of which RIP/VprBP is a member, associated with damaged-DNA specific binding protein 1 (DDB1; see Figure 1) (Angers et al., 2006; He et al., 2006; Higa et al., 2006; Jin et al., 2006). This family of proteins interacts with, and confers substrate specificity to Cullin 4- and DDB1-based E3 ubiquitin ligase complexes (hereby referred to as Cul4-DDB1 E3 ligases). After the discovery of this protein family, RIP/VprBP was accordingly renamed DDB1- and Cullin 4A-associated factor (DCAF) -1.

Recently, several laboratories reported that Vpr associates with a Cul4A-containing E3 ligase complex via its association with DCAF-1 and/or DDB1 (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). Knockdown of DCAF1 by RNA interference prevented Vpr co-immunoprecipitation with DDB1. These data form a model in which DCAF1 bridges Vpr to DDB1 and the larger E3 ligase complex. Based on this model, at least two possibilities could explain Vpr-induced G₂ arrest: 1) Vpr directs the Cul4-DDB1 E3 ligase complex toward degradation of a cellular protein; or 2) Vpr inhibits the function of the Cul4-DDB1 E3 ligase. If the first possibility is correct, then two types of Vpr mutants should exist: Those that ablate DCAF1 binding, and those that ablate substrate binding. Indeed, mutation of Vpr at Q65 (Q65R) prevents binding of Vpr to DCAF1, while the Vpr mutant R80A retains the ability to bind DCAF but does not induce G₂ arrest (presumably because it lacks the ability to bind the E3 ligase substrate). In addition, expression of Vpr R80A exerts a dominant negative (DN) effect (overrides G₂ arrest) when coexpressed with wt Vpr. However, mutation of Q65 (Q65R) in the context of R80A eliminates the DN effect of Vpr R80A, suggesting that the DN phenotype of Vpr R80A requires its ability to bind DCAF1 (Dehart et al., 2007; Le Rouzic et al., 2007).

In further support of a model in which proteosomal activity is required for Vpr-induced G₂ arrest, DeHart et al. demonstrated that inhibition of proteosomal activity either by pharmacological inhibition with epoxomicin or by overexpression of a dominant-negative ubiquitin mutant, Ub(K48R), abrogated Vpr-induced G₂ arrest (Dehart et al., 2007). Together, these data support a model in which Vpr directs the Cul4-DDB1 E3 ligase complex to ubiquitinate and degrade a cellular protein(s) required for cell cycle progression. Obviously, the elucidation of such a protein(s) is currently an area of intense investigation and we anticipate that its discovery will open new and exciting avenues for the development of anti-HIV-1 therapeutics.

Vpr and Apoptosis

HIV-1 infection and apoptosis

The precise mechanism by which CD4⁺ T cells are lost over the course of an HIV-1 infection is poorly understood (Hazenberg et al., 2000; Roshal et al., 2001). 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 (Finkel et al., 1995; Muro-Cacho et al., 1995). In contrast, Ho et al. and Wei et al. reported that HIV-1 replication directly leads to death of infected cells *in vivo* (Ho et al., 1995; Wei et al., 1995). 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, apoptosis caused by hyper-activation of the immune system, and apoptosis of infected cells induced by expression of viral genes (reviewed in (Roshal et al., 2001)). Given that these mechanisms are mutually exclusive, it is likely that a combination of them contributes to the decline in CD4⁺ T cells over the course of HIV-1 infection in the absence of treatment.

Apoptosis is a homeostatic process utilized by multicellular organisms to remove cells from tissue with minimal disturbance to tissue architecture or function. Cells utilize several apoptotic pathways that can be categorized as either extrinsic or intrinsic. Extrinsic apoptotic pathways involve ligation of death receptors such as Fas or TNF. The binding of death receptors to their cognate ligands leads to the recruitment of death domain-containing proteins such as FADD (Fas-activated death domain) and the apical caspase, caspase 8, to intracellular death receptor domains. From this platform, the proform of caspase 8 is cleaved and activated, at which point it can activate downstream caspases such as caspases 3 or 7, or cleave Bid to generate tBid,

which translocates to the mitochondria to promote Bax/Bak oligomerization, leading to cytochrome c release (reviewed in (Danial and Korsmeyer, 2004)).

In the intrinsic pathway, the apoptotic signal originates from within the cell, which occurs, for instance, in the case of DNA damage. In this pathway, pro-apoptotic signals triggered by the intracellular damage activate pro-apoptotic Bcl-2 family members such as Bad, Bim, and Puma, which promote Bax/Bak oligomerization. Oligomerization of Bax and Bak facilitates the release of cytochrome c, and other pro-apoptotic proteins from the intermembrane space. Once cytoplasmic, cytochrome c binds to Apaf-1, which serves as a co-factor to oligomerize and activate the initiator caspase 9 to promote cell death. Active initiator caspases cleave and activate the zymogenic forms of executioner caspases, such as Caspase 3. Executioner caspases cleave substrates throughout the cell to either dismantle cellular components, or activate other zymogens involved in the apoptotic process (reviewed in (Danial and Korsmeyer, 2004)).

Vpr activates the intrinsic apoptosis signaling pathway

As mentioned above, one key point of difference between the extrinsic and intrinsic pathways lies at the level of apical caspase activation (activation of caspase 8 is unique to the extrinsic pathway). In an attempt to determine which of these pathways is activated by Vpr, Muthumani *et al.* reported that Vpr-expressing cells underwent apoptosis via the intrinsic pathway, characterized by cytochrome c release, and caspase 9 (Muthumani *et al.*, 2002). Importantly, the authors did not observe activation of caspase 8, or expression of Fas or its ligand in response to Vpr (Muthumani *et al.*, 2002). These data ruled out the possibility that the Vpr-induced apoptotic signal was detected by death receptors on the cell surface, and was consistent with the possibility that Vpr induces apoptosis through intracellular stress.

Relationship between cell cycle arrest and apoptosis by Vpr

Because the most overt phenotype of Vpr *in vitro* is probably cell cycle arrest, the suspicion that cell cycle arrest and apoptosis by Vpr might be causally related has garnered attention in the literature. In support of model in which cell cycle arrest and apoptosis occur independently, Nishizawa *et al.* reported that mutation of certain C terminal amino acids, such as isoleucine 74, rendered Vpr moderately impaired for apoptosis but left its cell cycle arrest function intact (Nishizawa *et al.*, 2000). In addition, Waldhuber *et al.* reported that the Vpr-GFP fusion protein does not induce cell cycle arrest but effectively induces apoptosis (Waldhuber *et al.*, 2003). However, a recent report challenges these findings (Andersen *et al.*, 2006). Specifically, it was showed that Vpr mutated at isoleucine 74 induces apoptosis in immortalized CD4+ T cells at levels similar to wt Vpr. In addition, the Vpr-GFP fusion protein carrying an Arg to Ala mutation at position 80 of Vpr (a mutation that renders Vpr inactive for cell cycle arrest) still induced apoptosis, suggesting that apoptosis induced by Vpr-GFP may represent a gain-of-function phenotype unrelated to the nature of wild-type Vpr (Andersen *et al.*, 2006).

Early reports supporting a link between cell cycle arrest and apoptosis demonstrated a temporal relationship between the two, in which G₂ arrest peaked first, followed by apoptosis (Poon *et al.*, 1997; Stewart *et al.*, 1997; Stewart *et al.*, 2000). Later, in an attempt to identify a pathway to link the two phenotypes, Zhu *et al.* showed that treatment of cells with caffeine, an inhibitor of ATR and ATM, relieved both cell cycle arrest and apoptosis by Vpr (Zhu *et al.*, 2001). Later, the use of RNAi allowed for more specific targeting of proteins. Taking advantage of this, Yuan *et al.* showed that RNAi knockdown of Wee1, a Cdk1-inhibiting kinase downstream of ATR, alleviated both G₂ arrest and apoptosis induced by Vpr (Yuan *et al.*, 2004; Yuan *et al.*, 2003).

It was later found that siRNA-mediated depletion of ATR dramatically abrogated apoptosis by Vpr (Andersen *et al.*, 2006; Andersen *et al.*, 2005). In contrast, depletion of ATM, or its

downstream target, Chk2, had no effect. Andersen *et al.* also found that an essential downstream mediator of the pro-apoptotic signal initiated by ATR was the growth arrest and DNA damage protein 45 α (GADD45 α ; Figure 1) (Andersen *et al.*, 2005). It was also observed that BRCA1 was phosphorylated in an ATR-dependent manner at serine 1423 and that it formed BRCA1 nuclear foci (Andersen *et al.*, 2005; Zimmerman *et al.*, 2004). Indeed, the presence of activated BRCA1, a known p53-independent regulator of Gadd45 α , may help to explain how Vpr kills cells independently of p53 status. Therefore, from the above studies it was concluded that Vpr induced G₂ arrest and apoptosis induction are functionally linked, and both effects require the upstream contribution of the ATR kinase (Figure 1).

An alternative model to explain Vpr-induced apoptosis was proposed by Kroemer and colleagues. The authors found that recombinant Vpr or C terminal Vpr peptides associate with fractionated mitochondria via an interaction with the adenine nucleotide transporter (ANT), a component of the permeability transition pore that resides at the inner mitochondrial membrane (Jacotot *et al.*, 2000; Roumier *et al.*, 2002; Vieira *et al.*, 2000). The addition of C terminal Vpr to mitochondria triggered mitochondrial membrane permeabilization (MMP), which allows release of pro-apoptotic proteins harbored in the inner membrane space, such as cytochrome c (Jacotot *et al.*, 2000; Roumier *et al.*, 2002; Vieira *et al.*, 2000). Induction of MMP by Vpr was suppressed by overexpression of Bcl-2 (Jacotot *et al.*, 2000).

Recent studies of ANT and cyclophilin D knockout mice suggest that these mitochondrial pore components may promote necrotic cell death such as that induced by high cellular calcium concentration or oxidative stress, but not apoptotic death induced by DNA damage (Baines *et al.*, 2005; Kokoszka *et al.*, 2004; Nakagawa *et al.*, 2005). With the advent of RNA interference as a method of genetic manipulation, the role of ANT in Vpr-induced apoptosis was revisited (Andersen *et al.*, 2006). In these studies, siRNA mediated depletion of ANT did not appreciably affect Vpr-induced apoptosis, whereas depletion of another mitochondrial gateway protein, Bax (Figure 1), very effectively blocked apoptosis. In addition, it was found that induction of apoptosis by Vpr correlated with a conformational change in Bax that is considered synonymous with its activation (Andersen *et al.*, 2006). How can data supporting Vpr's interaction with ANT be reconciled with data supporting the requirement of pro-apoptotic stress pathways upstream of the mitochondria? Interestingly, C terminal Vpr has recently been shown to induce apoptosis in non-dividing differentiated monocytes (Mishra *et al.*, 2007). It is possible that the interaction with ANT may be relevant in cell types that display little or no cell cycle activity (macrophages, or neurons), while cells with high replicative activity (immortalized fibroblasts, or activated CD4⁺ T cells), may die more rapidly from cell cycle insults. It will be important to explore these potential differences in the future.

Cytostatic activity of Vpr in yeast

Soon after the cell cycle arrest activity of Vpr was documented in human cells, Zhao *et al.* reported a similar activity in fission yeast (*Schizosaccharomyces pombe*) (Zhao *et al.*, 1996), a model organism widely used in cell cycle studies. Overexpression of Vpr in yeast led to Cdc2 kinase inhibition and for most of the Vpr mutants examined, phenotypes were similar in mammalian and yeast cells (Chen *et al.*, 1999; Zhao *et al.*, 1996). These observations argued that Vpr targets an evolutionary conserved mechanism of cell cycle control. In this hypothesis, the existence of well-characterized yeast mutants and the relatively easy handling of fission yeast would be advantageous to further dissect Vpr-mediated cell cycle arrest as well as to set up large-scale screens for inhibitors of Vpr function. However, two lines of evidence may challenge the relevance of the fission yeast model in the study of Vpr activity. First, Vpr-mediated G₂ arrest in yeast occurs independently of classical DNA damage checkpoints and does not require the Chk1 kinase (Elder *et al.*, 2000; Masuda *et al.*, 2000; Matsuda *et al.*, 2006), in contrast to what has been observed in mammalian cells (Roshal *et al.*, 2003). Second,

the gene encoding DCAF1, which appears to be crucial for Vpr-mediated G₂ arrest in human cells, shows no obvious ortholog in *S. pombe*. However it is intriguing that during evolution the tandem Cul4-DDB1 appeared in fission yeast whereas it is absent in budding yeast (Liu et al., 2003), where overexpression of Vpr inhibits growth but does not cause G₂ arrest (Gu et al., 1997). Whether Vpr arrests *S. pombe* cell cycle by using a surrogate of DCAF1 to usurp the Cul4 ligase or by an unrelated mechanism remains an open question.

Conclusion

Recent progress has been made in understanding the basis for two of Vpr's most prominent functions: G₂ arrest and apoptosis. However, several issues remain to be solved. Specifically, clarification is needed to understand the nature of Vpr-mediated manipulation of the ubiquitin proteasome system. How does Vpr manipulate the cognate E3 Ubiquitin ligase it recruits? What is the target(s) whose degradation induces replication stress?. Another key issue will be to further validate the *in vitro* Vpr phenotypes in HIV-1 infected patients and determine whether targeting of these recently discovered pathways has the potential for a therapeutic benefit. As G₂ arrest appears to be the gateway to at least two of Vpr's additional functions (apoptosis, LTR transactivation), we propose that a therapy aimed at interfering with this cytostatic property of Vpr may offer clinical benefit.

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Figure 1.

Overview of the signaling pathways thought to be responsible for the cytostatic and pro-apoptotic effects of Vpr.