HIV-1, Vpr and the cell cycle

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The human immunodeficiency virus 1 (HIV-1) is a complex retrovirus with more genes than most retroviruses. One of these extra genes codes for a protein called Vpr, which has recently been shown to prevent activation of the mitotic cyclin-dependent kinase and thereby prevent infected cells from undergoing mitosis and proliferating. Vpr also plays an important role in another property of HIV-1 that is unusual for a retrovirus – its ability to enter the nucleus of a nondividing cell. Understanding the interactions between HIV-1 and the cell cycle should lead to new insights into both viral pathogenesis and basic cell biology.

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Introduction

Human immunodeficiency virus 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), is a member of the lentivirus class of retroviruses. Retroviruses are RNA viruses that use a viral enzyme, reverse transcriptase, to copy their genome into DNA which is then efficiently integrated into the host-cell genome. HIV-1 primarily infects activated CD4 T cells and terminally differentiated cells of the macrophage lineage. Progressive depletion of CD4 T cells is the defining feature of the immunodeficiency, whereas infection of brain macrophages and/or microglial cells is responsible for AIDS dementia [1] and infection of Langerhans cells in the macrophage lineage may be important for viral transmission [2–4].

Our understanding of HIV-1 pathogenesis has changed enormously in the last two to three years. Until recently, it was thought that the long period of latency between the time of infection and the onset of AIDS was associated with latency of the virus within infected cells. Now, however, we know that large amounts of virus are produced in the lymph nodes at all times [5,6]. Moreover, recent studies have shown that over 99 % of the virus in the periphery is produced by cells with a half-life of less than two days [7–9]. About 2×10^9 infected cells are killed and about the same number are replenished every day in an HIV-1-infected individual [7]. There is currently some debate about whether the replenishment of T cells in the periphery is due to regeneration or to redistribution [10]. Nonetheless, the overall picture is one in which virally infected cells are rapidly eliminated, but not before enough new virus is produced to sustain new rounds of infection. One hypothesis for HIV-1 pathogenesis is that, in the course of the disease, higher levels of virus lead to higher levels of cell death which eventually outrun the replenishment of T cells.

A second class of retroviruses, called oncoviruses because they are often oncogenic, differ from HIV-1 and the lentiviruses both in their genome structure and in their effects on infected hosts. HIV-1 is a complex retrovirus with a genome that includes nine genes (Fig. 1). With the exception of the human T-cell leukemia viruses, most oncoviruses, in contrast, are simple and have only three genes; they establish chronic infections in which the integrated provirus replicates with the host-cell genome as a permanent part of a chromosome, all the while continuing to produce virus. Indeed, vestiges of ancient integrations of oncoviruses into the germline genome, called endogenous viruses, mark the genomes of nearly all vertebrate species. In contrast to HIV-1, which causes destruction of

the productively infected host cells within a short time of infection, most oncogenic retroviruses have no deleterious effects on the growth or function of their host cell.

Given the different relationships HIV-1 and oncoviruses have with their host cells, it is not surprising to find that the interaction of HIV-1 with the host cell cycle is unlike that of many other retroviruses. Recent results, which I shall review here, show that a protein encoded by the HIV-1 genome and called Vpr — for viral protein R [11,12] — plays an important part in two unusual aspects of the interaction between HIV-1 and the host cell cycle. Vpr prevents infected cells from proliferating, by interfering with normal cell-cycle control, and it collaborates with the matrix protein (MA) to enable HIV-1 to enter the nucleus of a nondividing cells. Thus, the replication of HIV-1 and ultimately its pathogenesis are intrinsically tied to cell-cycle control.

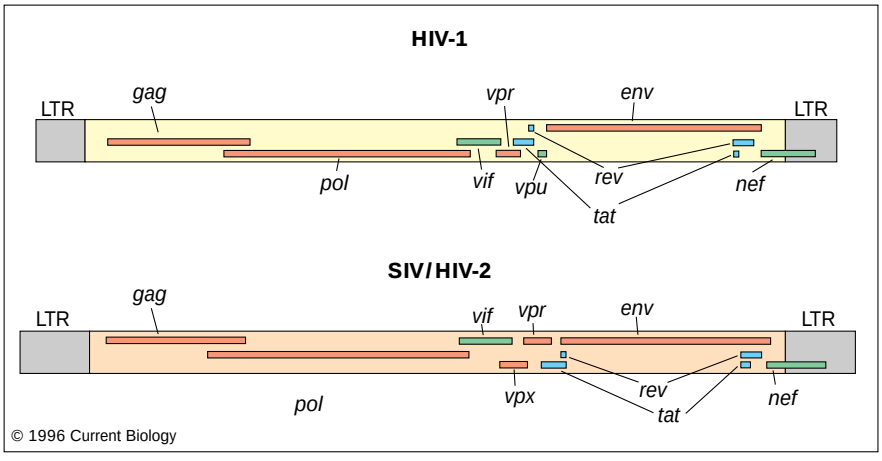
Vpr and cell-cycle control

Vpr is a 96 amino-acid (14 kDa) protein, encoded by a small open-reading frame, *vpr*, which overlaps the *vif* and *tat* genes at the 5′ and 3′ ends, respectively (Fig. 1). Among different HIV-1 isolates, *vpr* is one of the most conserved genes [13]. T cells infected in culture with an HIV-1 isolate that contains an intact *vpr* gene do not grow and eventually die [14–16]. Although it is possible to derive a culture of T cells that are chronically infected with HIV-1, in nearly all cases the virus particles produced contain mutations in *vpr* [15,17]. For example, the H9/HIVIIIb strain from Robert Gallo's laboratory, which has been in culture for over a decade, contains a frameshift mutation that truncates the predicted Vpr protein and renders it nonfunctional [13]. Cells expressing normal Vpr, from the complete HIV-1 genome or alone from an expression plasmid carrying *vpr*, do not proliferate but accumulate in the G2 phase of the cell cycle [15,18–21], before breakdown of the nuclear envelope and chromatin condensation [19,21]. Vpr thus prevents cells from passing into mitosis.

Vpr prevents mitosis in human and primate cells from a variety of tissues, as well as in infected T cells [15,18–21], but not in at least one mouse cell line [19]. It is cytostatic rather than cytotoxic, because T cells that express Vpr can be arrested in G2 for several days [21]; however, cells respond in different ways to this cytostatic effect. Some tumor cell lines differentiate as a result of the block to cell proliferation [22], whereas others reinitiate DNA synthesis leading to polyploidy [21]. The modest enhancement by Vpr of transcription driven by regulatory sequences in the viral long-terminal repeat (LTR) [23] may also be a result of cell-cycle arrest in G2, which would explain why Vpr can activate latent proviruses [24]. Eventually, T cells expressing Vpr die by apoptosis (I. Chen, personal communication). The consequences of Vpr expression are thus multifaceted, and might be expected to have complex effects on the host.

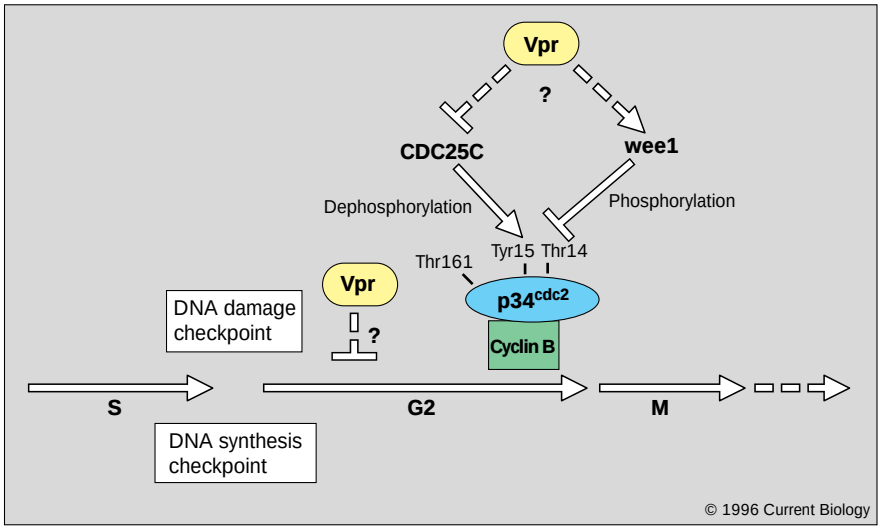
Passage of cells from G2 into mitosis is controlled by the mitotic cyclin-dependent kinase (Cdk) complex, a complex of the regulatory subunit cyclin B and the catalytic subunit p34^{cdc2} (Fig. 2; reviewed in [25]). The protein kinase activity of p34^{cdc2} depends on association with cyclin B, which is destroyed during mitosis and synthesized anew in late S phase and G2 . Phosphorylation of p34^{cdc2} on residue Thr161 by cyclin-activating kinase (CAK) is also necessary for mitotic Cdk activity. Although mitotic Cdk is produced, and phosphorylated by CAK, in early G2, the protein kinase wee1 prevents its premature activation by phosphorylating p34^{cdc2} on residues Thr14 and Tyr15; dephosphorylation of Thr14/Tyr15 by the protein phosphatase CDC25C eventually activates cyclin B–p34^{cdc2}, allowing progression to mitosis.

Figure 1



Genome organization of HIV-1 and SIV. Open reading frames that encode proteins which are efficiently incorporated into virions are in orange; those encoding regulatory proteins are in blue; and those encoding other auxiliary proteins are in green. The different vertical positions denote different reading frames. The long terminal repeats (LTRs), shaded grey, contain sequences necessary for transcriptional initiation and termination, integration and binding the viral transactivator Tat. The genomes of oncogenic retroviruses have only three genes: *gag*, *pol* and *env*.

Figure 2



Points at which Vpr might interact with the cell cycle to effect a G2-phase cell-cycle arrest. Arrowheads indicate activation and T-heads indicate inhibition; solid lines indicate direct interaction and dashed lines indicate indirect interaction. As explained in the text, wee1 is a kinase that inhibits cyclin B–p34^{cdc2} activity, and CDC25C is a phosphatase that activates it. Vpr probably acts upstream of CDC25C and wee1 by indirectly causing inhibition of CDC25C and activation of wee1. PP2A has been implicated in the phosphorylation control of CDC25C and wee1 (see text). It is also possible that Vpr acts downstream of a G2 checkpoint.

Cells that express Vpr have very low or undetectable levels of cyclin B–p34^{cdc2} kinase activity, although both cyclin B and p34^{cdc2} proteins are present [18,20,21]. The cyclin B–p34^{cdc2} kinase is inactive because of phosphorylation on Thr14 and Tyr15 [18–21]. It is unlikely that Vpr blocks the activity of the cyclin B–p34^{cdc2} kinase directly, because expression of a mutant p34^{cdc2} that cannot be inactivated by hyperphosphorylation, because of Thr14/Tyr15 mutation, overcomes the Vpr-induced G2 block [20]. Moreover, Vpr does not bind to cyclin B or p34^{cdc2} [18], and forced activation of cyclin B–p34^{cdc2} by treatment of cells with the phosphatase inhibitor okadaic acid overcomes the Vpr-induced G2 block [19]. Vpr therefore prevents cells from entering mitosis by interfering with a stage of the cell cycle that is upstream of cyclin B–p34^{cdc2} activation by dephosphorylation.

The wee1 kinase and CDC25C phosphatase that control the kinase activity of cyclin B–p34^{cdc2} are themselves controlled by phosphorylation. In cells that express Vpr, CDC25C is in its inactive, hypophosphorylated state [18], and wee1 is in its active hypophosphorylated state (W. Greene, personal communication). It is likely, therefore, that Vpr acts upstream of these enzymes to affect their coordinate regulation (Fig. 2). Activation of the DNA damage-induced checkpoint control in G2 would also have these effects, but it is unlikely that Vpr causes DNA damage because the arrest in G2 by Vpr is unaffected by the presence or absence of the p53 and ataxia telangectasia (AT) proteins, which mediate a checkpoint that arrests the cell cycle in response to damaged DNA, and is qualitatively different from the DNA-damage checkpoint control in its response to methylxanthines [21]. These results do not, however, rule out the possibility that Vpr acts downstream of checkpoint controls on either DNA damage or DNA synthesis (Fig. 2). In addition, because of its involvement with protein import into the nucleus (see below), it is possible that Vpr interferes with the transition from G2 to mitosis by interfering with the intracellular localization of a rate-limiting protein during G2.

Various cellular proteins bind to Vpr, but none fully explains Vpr's biological effects. The transcription factor Sp1 [26] and a 41 kDa cytosolic protein that coprecipitates with the glucocorticoid receptor [27] were shown to bind Vpr by direct binding assays. The cellular protein uracil DNA-glycosylase (UNG), which initiates removal of uracil from DNA that has been formed by incorporation of dUTP or by deamination of cytosine, was found to bind to Vpr [28]. However, *Saccharomyces cerevisiae* cells that lack UNG are viable, and no cell-cycle defect is caused by either under- or over-expression of UNG [29,30], so UNG has no obvious involvement in the cell cycle. Lastly, an intriguing interaction has been found between the 65 kDa regulatory subunit of the enzyme protein phosphatase 2A (PP2A) and Vpr (W. Greene, personal communication). PP2A is a protein serine/threonine phosphatase that plays many roles in cell metabolism. Among these roles is the regulation of the transition from G2 to mitosis through the dephosphorylation of CDC25C and wee1 [31]. Interaction of Vpr with PP2A could, therefore, play a role in causing constitutive activation of wee1 and inactivation of CDC25C.

A major reason to remain cautious about data implicating various Vpr binding partners is simply that there are so many of them. In addition to the proteins already mentioned, direct binding, yeast two-hybrid and genetic experiments have also indicated that Vpr binds to itself (R. Benarous, personal communication) and to at least three places on the HIV-1 Gag polyprotein [32–34]. It will

be a challenge to determine which are the biologically relevant interactions. Because the proliferation of both *S. cerevisiae* [35] and *Schizosaccharomyces pombe* [36] cells are also inhibited by Vpr, studies in these genetically tractable organisms may help determine the roles that interaction of Vpr with other host-cell proteins play in cell-cycle control. *S. pombe* appears the better model system, because Vpr arrests this type of yeast cell in G2 phase of the cell cycle [36], whereas the block to proliferation in *S. cerevisiae* cells is less clear.

Infection of terminally differentiated cells

Infection of T cells by HIV-1 requires cell activation and exit from the quiescent, G0 stage of the cell cycle [37–39]. However, HIV-1 can also infect terminally differentiated and other metabolically active nondividing cells [40–42]. This ability of HIV-1 to infect terminally differentiated cells seems unusual, because it has long been known that oncoviruses cannot integrate into the genomes of nonproliferating cells [43,44]. Passage of cells through mitosis is necessary for productive infection by oncoviruses because entry of their proviral DNA into the nucleus occurs only during mitosis [45,46].

Although one study argues that HIV-1 infects only proliferating macrophages [47], others have shown that some strains of HIV-1 replicate in terminally differentiated macrophages and in stimulated peripheral blood lymphocytes with similar kinetics [48]. It is unlikely, therefore, that efficient growth of HIV-1 in macrophages can be explained by proliferation of a minor population of cells in the macrophage cultures. Furthermore, HIV, but not oncogenic retroviruses, can efficiently infect cells that are artificially arrested in the cell cycle [42,46]. Lastly, pseudotyped retroviral vectors made from HIV-1 can infect neurons and astrocytes in rat brains — cell types that do not divide and therefore cannot be infected with oncoviral vectors [49].

The ability of HIV-1 to infect nondividing cells is presumably related to the ability of the HIV-1 preintegration complex to enter the nucleus of host cells during interphase (reviewed in [50]). The preintegration complex is defined as the RNA–protein complex in infected target cells that is competent to integrate into the host genome (Fig. 3). There are at least two karyophilic proteins in the HIV-1 preintegration complex — the MA protein [51,52], and Vpr [53]. The MA in the preintegration complex that enters the nucleus is a minor form of the protein that is phosphorylated on serines and/or the carboxy-terminal tyrosine residue [54,55]. There appear to be two distinct mechanisms by which HIV-1 can enter into the nucleus (Fig. 3). An importin-dependent pathway is partially mediated by the MA protein, and an importin-independent pathway is mediated by Vpr [56]. These two independent nuclear-entry pathways (Fig. 3) explain why Vpr is functionally redundant with MA in allowing HIV-1 infection of nondividing cells [53,56].

The finding that Vpr is important for infection of nondividing cells and macrophages by HIV-1 is well-documented [57–59]. However, some controversy surrounds the mechanism. One recent report [49] showed that Vpr expressed *in trans* would permit a mutant HIV-1 that lacked the *vpr* gene and encoded a mutant MA protein to infect nondividing cells, as one might expect if Vpr promotes entry into the nucleus; but another [58] found that Vpr failed to act *in trans* to assist macrophage infection. The difference between these results is difficult to explain, but might be due to differences in levels of expression of Vpr or to different *vpr* alleles. In addition, there may be other effects of Vpr on virus replication in macrophages that are independent of nuclear entry. Given the number of secondary effects that Vpr can have in T cells, this would not be altogether surprising.

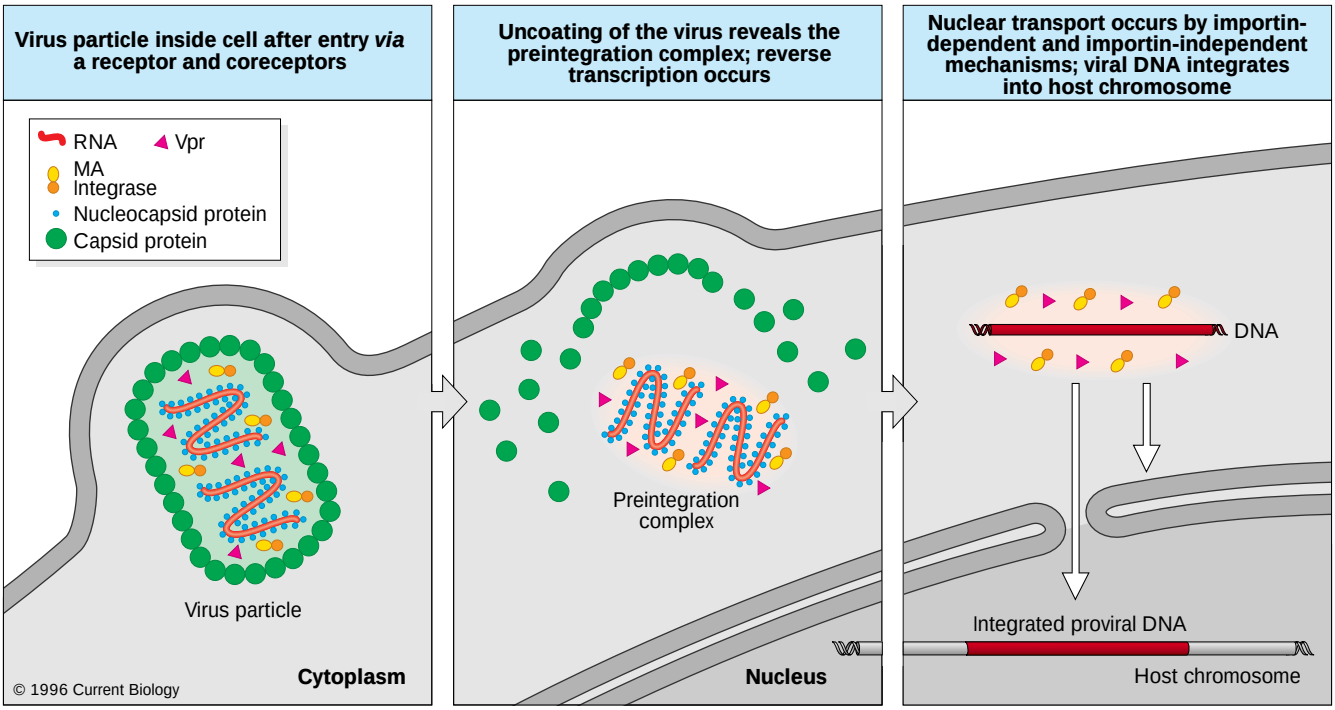
The quantitative effects of mutations in the MA nuclear localization sequence (NLS) and Vpr on virus growth in macrophages can also be variable [60]. This is probably explained, however, by the presence of at least one additional protein in the preintegration complex, besides MA and Vpr, which works better in some systems than in others to mediate nuclear entry [49,56]. Adaptation to growth in a variety of conditions may have selected for multiple redundant mechanisms of nuclear entry. The extent to which these different mechanisms are important in various nondividing cell types has yet to be explored.

Vpr localization in host cells

Vpr segregates into two compartments in virally infected cells: it is efficiently and specifically incorporated into virions by a process that is dependent on the p6 domain at the carboxyl terminus of the Gag precursor polyprotein [61–64], and it also accumulates in the nucleus [65,66]. It is not clear how Vpr itself gets into the nucleus, or how it influences entry of the preintegration complex. Vpr has no amino-acid motif that resembles a typical NLS, but two regions of the protein that are predicted to form amphipathic α helices appear to be important for accumulation of the protein in the nucleus [66,67]. Vpr enters the nucleus by a different mechanism than the importin-dependent pathway, because it is not blocked by a dominant-negative mutant of importin-α, which docks other NLS-containing proteins to the nuclear pore complex; nuclear entry of Vpr is also not competed by an excess of NLS-containing peptides [56].

Although the Vpr monomer is small enough to diffuse into the nucleus passively, Vpr forms oligomers that probably would not [68]. Vpr itself does not target reporter proteins such as β-galactosidase into the nucleus when introduced into cells as fusion proteins [67]; however, our recent data

Figure 3



The HIV-1 preintegration complex and nuclear entry. MA protein, Vpr, nucleocapsid protein, integrase and reverse transcriptase are inside the core with the viral RNA. After entry into cells, the viral core comes apart (uncoating), and the preintegration complex enters the nucleus by either an importin-dependent process mediated by MA protein – and another, as yet unidentified viral protein – or an importin-independent process mediated by Vpr. The stoichiometry of proteins in the preintegration complex is not known. Reverse transcription is initiated but may not be complete by the time that nuclear import occurs, and it is not known if the components of the preintegration complex change during this process.

suggest that Vpr can target β-galactosidase to the nuclear membrane (my group's unpublished data). It is possible, therefore, that Vpr-mediated entry of the preintegration complex into the nucleus bypasses the need for importin by either directly or indirectly linking the preintegration complex to the nuclear membrane, whereupon other mechanisms mediate its entry through the pore (Fig. 3). Other Vpr-binding proteins, such as UNG, might then be responsible for retaining Vpr in the nucleus. A novel mechanism of nuclear entry has also been described for U2 snRNP [69].

How does cell-cycle arrest aid viral replication?

It is not clear why HIV-1 encodes a protein that prevents its host cells from proliferation. Many other viruses either increase the proliferation of their host cells or prevent their cell-cycle arrest by interfering with checkpoint control [70–72]. Experiments with the SCID–Hu mouse model, in which HIV-1-infected thymocytes are implanted into a genetically immunocompromised mouse, have failed to define a role for Vpr [73]. However, this model differs from HIV-1 infection of humans in the proliferative status of the infected cells and in the absence of an immune response to the infection, which may be important for Vpr to have an effect (see below). On the other hand, viruses such as HIV-1 that mutate and replicate rapidly evolve quickly, so that changes in the viral genome that increase fitness only minimally are rapidly fixed in the population, whereas slight replicative disadvantages are rapidly selected against [74,75]. It is therefore very unlikely that cell-cycle arrest by Vpr would be maintained if it were not beneficial to virus replication. The fact that the ability to cause G2 arrest is conserved among diverse species of lentiviruses [76] also argues that it must give some selective advantage.

Several hypotheses might explain how prevention of cell proliferation could benefit virus growth. First, Vpr's ability to cause cell-cycle arrest in G2 may be required for it to allow infection of nondividing cells. This is unlikely, however, because these two functions are segregated into separate proteins, Vpr and Vpx (Fig. 1), in the simian immunodeficiency viruses (SIV) and HIV-2. The *vpx* genes of SIV and HIV-2 have no role in cell-cycle arrest, but are required for efficient infection of terminally differentiated macrophages, whereas the *vpr* gene causes cells to accumulate in G2 but has no role in infection of macrophages [18,67,76–79]. The ability of Vpx to allow SIV infection of nondividing cells is related to its ability to allow the preintegration complex to enter the nucleus [79].

Because the ability to infect nondividing cells, and the ability to cause cell-cycle arrest in G2 are unlinked in SIV and HIV-2, they are probably are also unlinked in HIV-1.

Another hypothesis to explain a selection for Vpr-induced G2 arrest is based on the observation that cytotoxic T lymphocyte (CTL)-induced apoptosis is dependent on premature activation of the mitotic Cdk [80]. When activation of the cyclin B–p34^{cdc2} kinase is prevented by overexpression of wee1 (Fig. 2), cells are relatively protected from apoptosis mediated by granzyme B, a protease secreted from CTLs [81]. Because Vpr also prevents activation of the cyclin B–p34^{cdc2} kinase, it is possible that Vpr has evolved to prevent activation of the mitotic Cdk in order to prolong the life span of HIV-infected cells in face of the large CTL response *in vivo*. This hypothesis might help explain why HIV-1 infection is persistent and cannot be cleared by a seemingly robust CTL response [82].

A separate, but not mutually exclusive, hypothesis is that the selection for Vpr is to maintain infected cells in G2, because expression of viral genes is highest in this phase of the cell cycle. Because infected cells have an average life span of only 2.2 days [8], it may be better for the virus to block the cells in a stage of the cell cycle when expression of viral genes is maximal. For example, we find that both virus production and expression of viral RNA are increased in G2 phase relative to G1 phase (my group's unpublished data). Any hypothesis to explain the functional significance of cell cycle arrest in HIV-1 infection must take into account the selective pressure for growth in the face of rapid elimination of infected cells *in vivo* [7–9]. Viral manipulation of the cell cycle might be one way to maximize viral expression in the brief time that virally infected cells survive.

Either of the hypotheses described above would be consistent with data from studies of SIV-infected macaques that show that deletion of *vpr* attenuates, but does not abolish, viral pathogenicity [83,84]. That is, *vpr* gives the virus an incremental selective advantage in overall growth, but is not absolutely required for either virus growth or pathogenicity. Deletion of both *vpr* and *vpx* in SIV, however, virtually eliminates virus replication in animals [80]. The Vpr protein of HIV-1, which combines the functions of the SIV proteins Vpr and Vpx (Fig. 1), may thus be crucial to viral replication in humans.

Future studies should concentrate on the major questions of how and why HIV-1 affects the cell cycle, and how cell-cycle arrest influences the viability of infected cells and virus production. An understanding of how HIV-1 infects nondividing cells may lead to a new generation of vectors for gene therapy. Furthermore, an understanding of the roles of Vpr in cell-cycle control and in the pathogenesis of AIDS may ultimately aid the design of antiviral therapies based on inhibition of Vpr function, and novel strategies to inhibit the proliferation of tumor cells.

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