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YEAST PERSPECTIVES ON HIV-1 VPR

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TABLE OF CONTENTS

1. ABSTRACT

Increasing evidence suggests that HIV-1 viral protein R (Vpr) plays an important role in viral pathogenesis, as its functions are being linked to viral activation, suppression of human immune functions and depletion of human CD4 lymphocytes, which are the major clinical manifestation of AIDS. *In vitro*, Vpr shows multiple activities both in mammalian and yeast cells, which include nuclear transport, induction of cell cycle G2 arrest, morphological changes and cell death. The occurrence of these activities in yeast indicates that Vpr interacts with highly conserved cellular processes to cause these effects and allows Vpr activities to be studied in these genetically well characterized organisms. Studies of Vpr in fission yeast (*Schizosaccharomyces pombe*) and budding yeast (*Saccharomyces cerevisiae*) have helped to establish these major conclusions. 1) Vpr induces G2 arrest through inhibitory phosphorylation of the cyclin-dependent kinase by a pathway in which protein phosphatase 2A plays an important role. 2) Vpr fulfills its essential role in the nuclear transport of the viral pre-integration complex by binding to a novel site on importin α. 3) Vpr induces apoptosis by directly permeabilizing the mitochondrial membrane. 4) Vpr also appears to kill cells by mitochondrial-independent mechanisms. 5) G2 arrest and cell death induced by Vpr are two independent functions, and 6) amino acid residues of Vpr at position 29, 33 and 71 are important sites for maintaining the overall structure of Vpr. Future studies of Vpr in yeast are expected to make additional contributions to understanding the mechanisms of Vpr activities and may also help address the importance of these activities during the course of a HIV-1 infection.

2. INTRODUCTION

Protein R (Vpr) of human immunodeficiency virus type 1 (HIV-1) is a virion-associated viral gene product with an average length of 96 amino acids, and a molecular weight of approximately 15 kD. Vpr is a highly conserved viral protein among HIV, simian immunodeficiency viruses (SIV) and other lentiviruses (1, 2), suggesting an important function or functions for this protein. Increasing evidence suggests that HIV-1 Vpr plays a pivotal role in viral pathogenesis, as its functions are being linked to viral activation (3), suppression of immune functions (4), and depletion of CD4 lymphocytes (5). Studies on chimpanzees and an accidentally infected laboratory worker demonstrated that Vpr is required *in vivo* for viral pathogenesis as the mutant *vpr* gene in the virus initiating the infection inevitably reverted back to the wild-type Vpr (6).

Somewhat surprisingly for a small protein of 96 aa, *in vitro* studies indicate that Vpr has multiple activities. These activities include nuclear transport of the viral pre-integration complex (PIC) (7), induction of cell differentiation and cytopathic effects (8), cell cycle G2 arrest (9), and cell killing (5). The essential role of Vpr in the transport of the viral PIC into the nucleus is required for effective infection of non-dividing cells such as monocytes and macrophages (7, 10, 11). The nuclear transport property of Vpr appears to be highly conserved since Vpr localizes onto the nuclear envelope in a variety of eukaryotic organisms including not only mammalian but budding and fission yeast cells (11-13). Vpr blocks cell cycle progression by arresting cells in the G2 phase of the

Table 1. Homologues of yeast and human proteins that are related to HIV-1 Vpr activities.

Fission Yeast (*S. pombe*)	Budding Yeast (*S. cerevisiae*)	Human (*H. sapiens*)	Protein functions and Comments
Cell cycle G2/M control			
Cdc2	CDC28p	CDK1	Cyclin B-dependent kinase
Wee1	WEE1p	WEE1	Tyrosine kinase
Cdc25	CDC25p	CDC25C	Tyrosine phosphatase
PP2A	PP2A	PP2A	Protein phosphatase 2A
DNA damage and replication checkpoints			
Rad1	RAD17p	Hrad1	Nuclease
Rad3	ESR1 (MEC1/SAD3)	ATR	Caffeine-sensitive, DNA-activated protein kinase
Rad9	----	Hrad9	3'-5' Exonuclease
Rad17	RAD24p	Hrad17	Unknown (activates p53)
Rad24/25	BMH1p/2p	14-3-3	Nuclear export (Binds to phosphorylated Cdc25)
Hus1	----	Hhus1	A PCNA-related protein
Chk1	CHK1	CHK1	Kinase
Cds1	RAD53p	CHK2	Kinase
Nuclear transport			
SpSrp1 /Cut15	Srp1p	HSrp1/Qip1/Rch1	importin α
Vpr-binding proteins			
Rhp23*	Rad23**	HHR23A/B	Excision DNA repair enzyme
Spung1*	UNG1**	UDG	Uracil-N-glycosylase

Note: "---", not found; "*", our unpublished data. "**", interaction with Vpr has not yet been established.

cell cycle in both mammalian and fission yeast cells (9, 14-16), indicating another highly conserved function of Vpr. The G2 arrest induced by Vpr is thought to suppress immune functions by preventing T cell clonal expansion (4, 5) and providing an optimized cellular environment for maximal levels of viral replication (6). The cytopathic effects of Vpr were first described by Levy *et al.* (3). Expression of Vpr in a rhabdomyosarcoma cell line induced cell differentiation, as characterized by gross enlargement and altered morphology. Consistent with these observations, Vpr altered cell morphology in numerous other mammalian cell lines and yeast cells (14, 17-20). A fourth activity of Vpr is cell killing which may contribute to CD4 T-cell depletion in HIV-infected patients (5). This cell killing activity of Vpr is also highly conserved as it extends to all cells tested including human and yeast (14, 18, 20).

These four activities of Vpr are thus highly conserved since they are observed in cells over a large evolutionary distance ranging from human to yeast. This functional conservation indicates that Vpr most likely causes these effects by interacting with highly conserved cellular processes and provides the opportunity to study these Vpr activities in fission yeast *(Schizosaccharomyces pombe*) and budding yeast (*Saccharomyces cerevisiae*). Both of these unicellular eukaryotes with their ease of genetic analysis and small, completely sequenced genomes have been favorite model systems for basic processes of eukaryotic cells (for reviews see 21-24).

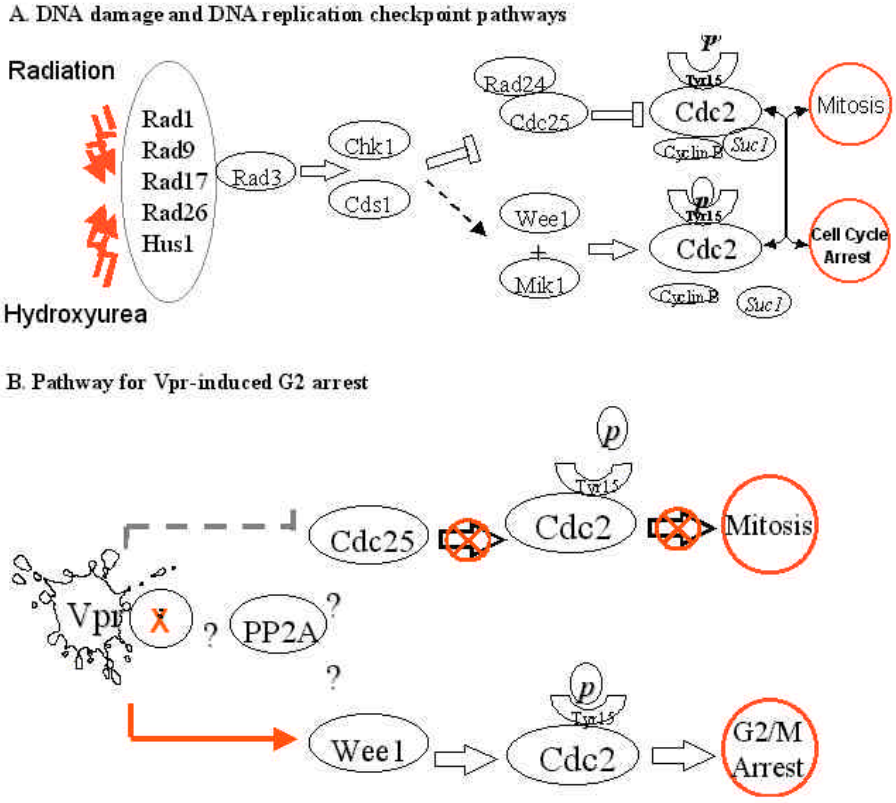
In this review, we will first summarize the current models for nuclear localization and induction of G2 arrest, where much of the models are based on studies in yeast, and of apoptosis where yeast studies are providing insights into the early steps of this process. We will then describe developing ideas about how Vpr interacts with these cellular processes and the contributions that studies in yeast are making to understanding Vpr activities.

3. CELL CYCLE G2 ARREST

3.1. Conservation of checkpoints for G2 arrest

Control of the cell cycle is one of the best examples of how studies in yeast can provide the basis for understanding the equivalent process in higher eukaryotic cells (25). The conservation of cell cycle controls between human and *S. pombe* has been clearly demonstrated by identification of human cognates for most of the *S. pombe* mutants in the G2 control pathways (Table 1). figure 1A shows a major pathway for the control of the G2 to M transition in fission yeast and human cells (23, 26). The cyclin-dependent kinase is inhibited by phosphorylation of Tyr15 for Cdc2 in fission yeast and Thr14 and Tyr15 for Cdk1 in human cells. These sites are phosphorylated by the Wee1 kinase during late G2 and are rapidly dephosphorylated by the Cdc25 phosphatase to trigger entry into mitosis. The G2 to M transition of budding yeast is regulated differently since inhibitory phosphorylation of the cyclin-dependent kinase Cdc28 of budding yeast does not play a major role in this transition (27, 28). Thus, fission yeast, but not budding yeast, is a suitable model system for studying G2 controls operating through inhibitory phosphorylation of the cyclin-dependent kinases.

Studies of DNA damage and DNA replication checkpoints illustrate how useful fission yeast is as a model system for G2 controls (23). DNA damage by radiation or



Fgure 1. Cell cycel G2 controls. (A) DNA damage and DNA replication checkpoint pathways. (B) Working model for Vpr-induced G2 arrest in fission yeast.

inhibition of DNA synthesis by hydroxyurea lead to G2 arrest in fission yeast. These two checkpoints both function by increasing the inhibitory phosphorylation of Tyr15 on Cdc2, and a strain with mutant Cdc2 changing Tyr15 to a nonphosphorylable Phe does not arrest in G2 in response to DNA damage or inhibition of DNA replication (29, 30). The pathway for the checkpoints shown in figure 1A is based on extensive genetic and biochemical analysis of fission yeast (23). The early genes in both checkpoint pathways, which include *rad1*, *rad3*, *rad9*, *rad17*, *rad26* and *hus1*, are thought to detect the DNA damage or incomplete DNA synthesis and lead to the phosphorylation of the Chk1 or Cds1 kinases (31, 32). The activated Chk1 or Cds1 kinase then directly phosphorylates the Cdc25 phosphatase (33, 34). The phosphorylated Cdc25 binds the Rad24 protein, and this complex is transported out of the nucleus to render Cdc25 inactive (35). These checkpoint pathways defined in fission yeast also apply in all major aspects to human cells where inhibitory phosphorylation of Cdk1 is essential for the checkpoints, and human homologues having been identified for all of major fission yeast genes in the pathways including the Chk1 and Cds1 kinases (23, 36, 37) (Table 1).

3.2. Vpr-induced G2 arrest through a PP2A-dependent pathway

Vpr induces G2 arrest through inhibitory phosphorylation of Cdc2/Cdk1 both in human and fission yeast cells. Three main lines of evidence support this conclusion. 1) Vpr induces hyperphosphorylation of Cdc2/Cdk1 (14, 38, 39). 2) Expression of nonphosphorylatable Cdc2/Cdk1 mutants, A14T F15Y of Cdk1 and F15Y of Cdc2, prevents Vpr-induced G2 arrest (38, 40). 3) Overexpression of the Cdc25 phosphatase reduces Vpr-induced G2 arrest (45; Elder et al., submitted). Consistent with the essential role of inhibitory phosphorylation of Cdc2/Cdk1 in Vpr-induced G2 arrest, Vpr does not induce G2 arrest in budding yeast where inhibitory phosphorylation does not control the G2 to M transition (27, 28, 41). Since Vpr induces G2 arrest through inhibitory phosphorylation of Cdc2/Cdk1 both in human and fission yeast cells, fission yeast is an excellent model system to elucidate the underlying mechanisms for Vpr-induced G2 arrest.

Given that the DNA checkpoints and Vpr both induce G2 arrest through inhibitory phosphorylation of Cdc2/Cdk1, Vpr might induce G2 arrest through a checkpoint pathway. This possibility has been thoroughly evaluated in fission yeast by expressing *vpr* in mutant fission yeast strains defective in early and late steps of the checkpoint pathways. None of the early checkpoint-specific mutants (*rad1*, *rad3*, *rad9* and *rad17*) have a significant effect on the induction of G2 arrest by Vpr (40). Furthermore, mutations in both *chk1* and *cds1*, which are thought to be the last steps specific for the checkpoint (33,

42, 43), also do not block Vpr-induced G2 arrest (Elder et al., submitted; 44). Therefore, Vpr does not use the checkpoint pathways to induce G2 arrest in fission yeast.

More limited data in human cells tend to support the conclusion that Vpr does not induce G2 arrest through the checkpoints pathways. Vpr still induced G2 arrest in cells from patients with ataxia telangiectasia (AT) (45). These AT cells are mutant for the human homologue of fission yeast Rad3 and do not arrest in G2 in response to DNA damage (36, 46). Thus, Vpr does not use this early part of the DNA damage checkpoint to induce G2 arrest in human cells. However, Poon *et al.* (105) have suggested that Vpr does induce G2 arrest through the DNA damage checkpoint in human cells based primarily on studies with pentoxifylline (PTX), a methyl xanthine. PTX prevents G2 arrest after DNA damage (47), and Poon *et al.* (105) found that PTX also inhibits Vpr-induced G2 arrest. PTX also inhibits Vpr effects including G2 arrest in fission yeast (20, Elder et al., submitted). However, since PTX inhibits Vpr-induced G2 arrest in fission yeast where the DNA damage checkpoint apparently plays no role, PTX inhibition alone is not strong evidence that the DNA damage checkpoint has a role in Vpr-induced G2 arrest.

Since Vpr does not appear to use the checkpoint pathways in fission yeast or human cells, what pathway does Vpr use to induce G2 arrest? A clue comes from studies with okadaic acid (OA), a potent inhibitior of protein phosphatase 2A (PP2A) (48, 49). Both in fission yeast and human cells, OA inhibits the induction of G2 arrest by Vpr (14, 39). This further similarity between Vpr-induced G2 arrest in fission yeast and human cells suggests that Vpr induces G2 arrest by activating PP2A. This possibility is supported by the observation that PP2A is known to play a role in cell cycle control both in fission yeast and higher eukaryotes (49, 50). Further evidence that PP2A has a role in Vpr-induced G2 arrest comes from the finding that mutations in genes (*ppa2* and *pab1*) coding for the catalytic and regulatory subunits of the fission yeast PP2A partially blocked Vpr-induced G2 arrest (44; Elder et al., submitted). Furthermore, an elevated PP2A protein level was observed in cell extracts isolated from the *vpr*-expressing cells, confirming that Vpr up-regulates PP2A in fission yeast cells (Elder et al., submitted). Recently, Hrimech *et al.* (106) have presented evidence that Vpr activates PP2A in human cells. Thus, activation of PP2A plays a major role in Vpr-induced G2 arrest both in fission yeast and human cells.

PP2A is thought to affect the cell cycle by acting on the Wee1 and Mik1 kinases and the Cdc25 phosphatase which control phosphorylation of Tyr15 (49, 50). In fission yeast, expression of *vpr* in strains with mutations in these genes suggests that Vpr inhibits Cdc2 predominantly by activation of Wee1 with inhibition of Cdc25 playing a minor role. A working model for the novel regulatory pathway(s) that Vpr uses to induce G2 arrest in fission yeast is shown in figure 1B. Expression of *vpr* cells activates PP2A activity either by direct association with the PP2A enzyme complex or by association with an intermediate protein(s) X. A protein phosphorylation cascade (depicted as "?") including PP2A is probably ultimately responsible for activation of the mitotic repressor Wee1 by altering its phosphorylation levels, which in turn inhibits Cdc2 by Tyr15 phosphorylation. While Wee1 plays the major role in the induction of G2 arrest by Vpr, Cdc25 appears to play a minor role and to be inhibited by this proposed regulatory pathway. Although PP2A is a major part of this proposed regulatory pathway, it is not yet known whether PP2A is completely or only partially responsible for Vpr-induced G2 arrest. Other protein kinases and phosphatase may also be regulated by Vpr to induce G2 arrest in fission yeast.

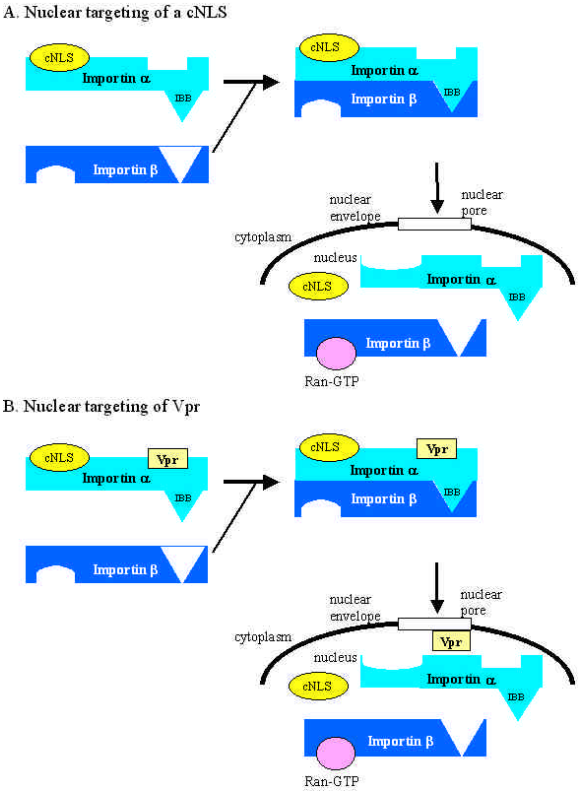
4. NUCLEAR LOCALIZATION

4.1. Importin a pathway for classical nuclear localization sequences (cNLS)

Figure 2A shows the model for nuclear transport of proteins containing a classical nuclear localization sequence (cNLS) (51, 52). The cNLS is a short amino acid region rich in Lys which binds to the adaptor protein importin α. The complex of cNLS-importin α then binds to the receptor importin β through the importin β-binding (IBB) region on importin α. Importin β interacts with components of the nuclear pore complex (NPC) as an essential part of the nuclear translocation process. The NPC is a large structure composed of 50 to 100 proteins called nucleoporins, which contains a central 10 nm aqueous channel through which proteins are actively transported. Once the cNLS-importin-α-importin β complex has been transported through the nuclear pore, RanGTP insures the directionality of the process. Only the nucleus has a high concentration of RanGTP, and binding of RanGTP to cNLS-importin α-importin β dissembles the complex and releases the protein carrying the cNLS into the nucleus. Importin α and importin β-RanGTP are then exported out of the nucleus to be reused in another round of nuclear transport. This model for cNLS translocation is partially based on work done in budding yeast, and the high degree of conservation is demonstrated by functional complementation of many budding yeast mutants in nuclear transport by human homologues (53).

4.2. Role of Vpr in nuclear transport

Nuclear localization of Vpr turns out to use a modification of the importin α pathway (reviewed in 54, 55). Most studies have reported that Vpr expressed without other viral proteins localizes predominantly to the nuclear envelope in human, fission yeast and budding yeast (11-13). Others have reported that Vpr localizes throughout the nucleus or to the mitochondria as described below. It was initially suggested from *in vitro* assays that Vpr did not use the importin α pathway since addition of excess cNLS did not inhibit the nuclear localization of Vpr (56, 57). However, further studies showed that Vpr does bind to importin α both from human and budding yeast, but the binding site is different from the binding site for cNLS (figure 2B) (11, 58, 59). Vpr also binds to nucleoporins, and based on this interaction (11) suggested that Vpr might function directly as a receptor for nuclear transport in place



Fgure 2. Nuclear localization. (A) Nuclear targeting of a cNLS. (B) Nuclear targeting of Vpr.

of importin β. However, Popov *et al.* (59) showed that importin β was necessary for nuclear localization of Vpr and that importin α, importin β, cNLS and Vpr form a ternary complex. Thus, the model (figure 2B) best supported by the evidence at present is that Vpr binds to a previously unknown site on importin α, and this complex in turn binds to importin β which serves as the receptor for transport through the nuclear pore. The effect of Ran-GTP binding to importin β on the ternary complex has not been reported and it is not understood why Vpr is frequently observed to be at the nuclear envelope although this may be related to the binding of Vpr to nucleoporins (11, 60). One study found Vpr to be at the inside of the nuclear envelope suggesting that Vpr is transported through the pore but then stops at the inside of the nuclear pore rather than being released into the nucleus (11).

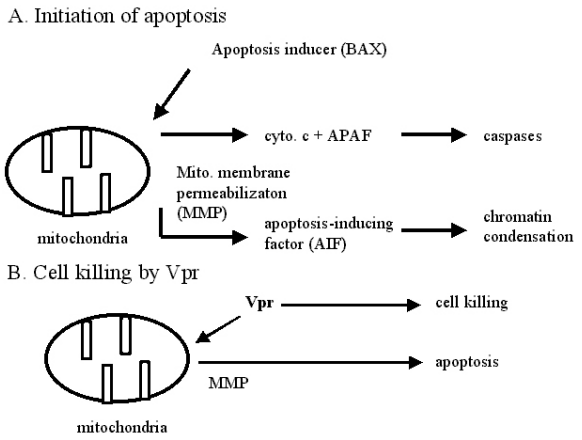
In the nuclear transport of the viral pre-integration complex (PIC), Vpr appears to assist transport by cNLS rather than functioning as an independent nuclear transport factor. There has been disagreement over the role and identity of cNLS in the nuclear transport of PIC, and at least one reason for this disagreement is the presence of two cNLS in the matrix protein (MA) (61). Deletion of either MA cNLS does not prevent nuclear translocation of PIC in macrophage cells (62, 63), but when both are deleted, nuclear transport of the PIC no longer occurs even when Vpr is present (61). Thus, Vpr does not function as an independent nuclear transport factor. However, when one or both MA cNLS are present in the PIC, nuclear transport occurs efficiently only when Vpr is present (10, 11, 58, 59) so Vpr plays an essential role in the nuclear transport of PIC. Vpr increases the binding of the MA cNLSs to importin α (58), and this increased affinity may be at least part of Vpr's role in nuclear transport. In general, the major role of Vpr may be in the nuclear import of usually large complexes since either MA cNLS when coupled as multiple copies to serum albumin gives transport into the nucleus without Vpr. However, the much larger viral PIC, which has multiple copies of both MA cNLS, is transported into the nucleus only when Vpr is present (54, 58, 61).

One interesting implication of a conserved Vpr-binding site present both on human and budding yeast importin α is that this binding site plays some important cellular function in nuclear transport and that a cellular protein binds to this site. Agostini *et al.* (64) have recently identified this cellular protein as Hsp70, a highly conserved heat shock protein, which competes with Vpr for binding to importin α. Hsp70 can in fact replace Vpr in the nuclear transport of PIC and similar to Vpr also strengthens the binding of MA cNLS to importin α (64). One cellular role of this Vpr/Hsp70 binding site thus appears to be in strengthening the interaction of a weak cNLS with importin α. The requirement for Vpr in the transport of the large PIC also suggests that this binding site on importin α might be required for efficient translocation of large complexes through the nuclear pore.

5. CELL KILLING

5.1. Role of mitochondria in apoptosis

It is becoming clear that mitochondria play a central role in the induction of apoptosis under many conditions where stress is placed on a mammalian cell (65, 66). Mitochondrial membrane permeabilization (MMP) by a variety of agents leads to the release of factors which initiate the apoptotic process (figure 3A), and cytochrome c is one of the principal apoptotic factors. When cytochrome c is released from the mitochondria, it combines with apoptosis-associated factor (APAF) in the cytoplasm to activate caspase 3 which in turn activates other caspases. These cysteine proteases are the downstream effectors of apoptosis which are responsible for some of the characteristics of the apoptotic cell. However, some aspects of apoptosis are caspase independent, and caspase inhibitors do not always prevent cell death after induction of apoptosis although these inhibitors may modify the apoptotic process (65, 66). One clear example of a caspase-independent activity is the apoptosis-inducing factor (AIF) (figure 3A). Like cytochrome c, this protein is also released from its normal location between the inner and outer mitochondrial membranes by apoptosis-inducing agents. However, unlike cytochrome c, caspases have no role in AIF activity, and after being released from the mitochondria, AIF moves to the nucleus where it induces one of the early characteristics of apoptosis, condensation of the chromatin at the periphery of the nucleus (67, 68). Thus, in many instances apoptosis is initiated in mammalian cells by factors



Fgure 3. Induction of apoptosis. (A) Role of mitochondria in initiating apoptosis. A number of apoptosis-inducing agents, for example BAX, act by permeabilizing the mitochondrial membrane to release factors which initiate apoptosis. (B) Cell killing by Vpr. One effect of Vpr is to permeablize the mitochondrial membranes by binding to ANT of the PTPC leading to apoptosis (84). There also appears to be a mitochondria-independent mechanism for cell killing by Vpr which acts by disruption of the cytoskeleton (14, 20, 41).

released from the mitochondria with both caspase dependent and independent steps downstream.

Even though yeast do not have caspases, they do undergo a process with some similarities to the apoptosis of mammalian cells, and this process is probably a simpler precursor to the more complex apoptotic process which evolved in mammalian cells (69-71). This apoptotic process in yeast is also initiated through MMP, and yeast are therefore being used as model systems for the mitochondrial dependent but capase-independent aspects of apoptosis. Much of the evidence supporting the existence of an apoptotic process in yeast comes from the study of mammalian proapoptic proteins expressed in yeast including Vpr which is discussed below. For example, three proapoptic proteins (Bak, Bax, and Ced4) which induce apoptosis in mammalian cells or nematodes also induce death in fission and budding yeast cells (72-75). Not only do these proapoptic proteins kill yeast, they also induce chromatin condensation, one of the nuclear changes characteristic of apoptosis in mammalian cells (76). Further similarities between the cell killing by Bax are that Bcl-2 and BI-1 inhibit the induction of apoptois by Bax both in yeast and mammalian cells (72, 73, 75). The first step in apoptosis is the also the same since Bax causes MMP to initiate cell killing both in mammalian and yeast cells. It has recently been reported that acidification of the cytosol by the H+ released from the mitochondria is important to the induction of apoptosis both in budding yeast and mammalian cells (77). Thus, yeast provides a good model system for studying the role of MMP in apoptosis.

5.2. Mitochondrial-dependent cell killing by Vpr

Most studies have reported that Vpr induces apoptosis in human cells (5, 78-81). Vpr expression thus leads to Annexin V binding (5), one of the early markers of apoptosis, and caspase inhibitors reduce the extent of cell killing by Vpr indicating that Vpr activates these downstream effectors of apoptosis (79, 80). However, there have been two reports that Vpr does not induce apoptosis but in fact functions as an inhibitor of apoptosis (82, 83). Both of these studies used stable transfectants constitutively expressing *vpr* at low levels, and the different results of these two studies may be related to the low level expression or to the selection of mutant cells resistant to apoptosis during the selection of stable transfectants. The five studies reporting induction of apoptosis expressed *vpr* at high levels, and it thus appears that these high levels of Vpr induce apoptosis in human cells.

Vpr also kills both budding and fission yeast, and the yeast cells show some of the characteristics expected for apoptotic cells (14, 18, 20). One strong similarity between fission yeast cells dying after expression of *vpr* is condensation of the chromatin around the periphery of the nucleus which is also seen at early stages of apoptosis in mammalian cells (20, 76). Purified AIF alone is capable of inducing chromatin condensation around the nuclear periphery in mammalian cells (67), and there is a conserved homologue of AIF in fission yeast (70). It is an interesting possibility that Vpr induces peripheral chromatin condensation through this fission yeast homologue of AIF.

With Vpr killing both human and yeast cells through an apoptotic process, it seemed likely that Vpr might be acting through the mitochondria by MMP. The first direct evidence for a mitochondrial role came from budding yeast. Macreadie *et al.* (1997) found that expression of *vpr* at intermediate levels did not kill budding yeast but did prevent growth on nonfermentable carbon sources such as glycerol. Yeast with mitochondria unable to carry out oxidative phosphorylation can grow on glucose, but are unable to grow on nonfermentable carbon sources so this finding indicated that *vpr* expressed at intermediate levels inactivated yeast mitochondria.

Jacotot *et al.* (84) have recently presented evidence strongly supporting the role of MMP in cell killing by Vpr both in human and yeast cells. Addition of extracellular Vpr to human cells or isolated mitochondria was shown to permeablize the mitochondria as indicated by measurements such as decreased membrane potential and the release of cytochrome c and AIF. The importance of this MMP to the induction of apoptosis was shown by inhibitors of MMP, such as Bcl-2, which reduced MMP and apoptosis. The major target for Vpr in the mitochondrial membrane is the permeability transition pore complex (PTPC) of which the two major components are ANT (adenine nucleotide translocator) and VADC (voltage-dependent anion channel), and Vpr was shown to bind specifically to ANT. Experiments in budding yeast provide further support for Vpr inducing cell death through MMP by interaction with PTPC. Externally added Vpr also kills budding yeast and the same region of Vpr is required to kill both yeast and mammalian cells. Mutant yeast strains deficient in ANT or VDAC showed reduced cell killing, and transformation with yeast ANT or human

VDAC restored the level of cell killing. Thus, MMP through interaction with the PTPC appears to be a major mechanism for cell killing by Vpr both in mammalian and yeast cells.

5.3. Mitochondrial-independent cell killing by Vpr

Although accumulating evidence indicates that MMP plays a role in cell killing by Vpr in mammalian and yeast cells, there are a number of reasons to think that there may be other mechanism(s) by which Vpr kills cells. First, Vpr seems to induce more cell death in human cells than can be accounted for by apoptosis alone (78). Second, as discussed below Vpr induces morphological changes which by themselves would seem to be lethal to the cell, and these morphological abnormalities not related in any known way to MMP. Third, the regions of Vpr required for MMP are sometimes different from the regions required for cell killing. For example, the studies on MMP showed that a peptide containing amino acids 52 to 96 of Vpr was sufficient to induce MMP and cell death when added to cells (84). However, while expression of wild-type *vpr* kills yeast, a *vpr* gene with a F34I mutation outside of this region did not kill either budding or fission yeast (12, 41). Fourth, most studies have reported that Vpr localizes to the nucleus (85-89) or nuclear envelope (11, 13, 60, 90, 91) in most cells raising the question of whether sufficient Vpr is present at the mitochondria to permeablize the membrane. Some studies have reported weak cytoplasmic labeling (13, 87) and Jacotot *et al.* (84) did demonstrate localization of Vpr to the mitochondria in a minority of cells. However, the persistence of different reported localization for Vpr, ranging from throughout the nucleus, to the nuclear envelope or to the mitochondria, strongly suggests that localization of Vpr is variable and depends on factors which remain to be clearly defined. Conditions which direct Vpr preferentially to the mitochondria would emphasize cell killing by MMP whereas conditions minimizing localization to the mitochondria would emphasize mitochondrial-independent pathways for cell killing.

These and other observations support the existence of cell killing by Vpr though mechanisms other than MMP, and these mitochondrial-independent pathways for cell killing by Vpr seem likely to involve the morphological changes induced by Vpr. Expression of HIV-1 Vpr causes cytopathic effects, as characterized by gross enlargement and altered cell morphology in a number of mammalian cell lines (8, 17, 92). These Vpr-induced morphological changes appear to be a highly conserved process as budding and fission yeast cells also changed morphology and became grossly enlarged when the *vpr* gene is expressed (14, 18). Further characterization indicated that alterations of cell wall biosynthesis and disruption of the F-actin cytoskeleton structure are some of the underlying causes of these morphological changes in yeast (20, 41).

One severe cellular abnormality induced by Vpr in fission yeast is altered chitin accumulation. In normal cells chitin is an essential element of yeast cell walls (93), and during cytokinesis, the septum that forms between the two new daughter cells has a large amount of chitin (94). After expression of *vpr*, one abnormality is that large amounts of chitin are deposited adjacent to irregular protruding structures that form on the cells (20). A second abnormality in chitin deposition is that multiple septa form in a single cell without nuclear division. Although the functional relevance of this particular effect to mammalian cells is at present unclear since mammalian cells do not have cell walls, this intriguing phenotype suggests that expression of *vpr* separates nuclear division and cytokinesis in fission yeast.

Vpr also disrupts the actin cytoskeleton in both budding and fission yeast (20, 41). The actin cytoskeleton in normal yeast cells directs growth of the cell, and reorganizations of the cytoskeleton are essential for the changes in cell growth during the cell cycle. For example, formation of an actin ring at the center of fission cells during mitosis is believed to direct where the septum will form (95, 96). Upon expression of *vpr*, these defined cytoskeletal structures are replaced by randomly oriented F-actin distributions. As the actin cytoskeleton is essential for viability of yeast (97), these severe disruptions would be expected to kill the cell independently of other effects of Vpr. The importance of this cytoskeletal disruption to cell killing by Vpr is strongly supported by the characteristics of a suppressor. Overexpression of the heat shock gene *hsp42* gene restores the normal actin cytoskeleton and eliminates cell killing by Vpr in budding yeast (41). This gene also stabilizes the actin cytoskeleton during heat shock suggesting that Hsp42 suppresses Vpr killing by directly acting on the F-actin cytoskeleton. Since there is no obvious connection to the mitochondria in disruption of the cytoskeleton by Vpr or in its suppression by Hsp42, it appears that this mechanism of cell killing is independent of MMP.

6. STRUCTURAL REQUIREMENTS AND INDEPENDENCE OF *VPR* ACTIVITIES

Studies of Vpr mutants have allowed correlations of activities with Vpr structure and suggested that some Vpr activities are independent of each other. A tertiary structure of Vpr proposed on the basis of NMR consists of a α helix-turn-α helix domain in the amino half from amino acids 17 to 46 and a long α- helix from aa 53 to 78 in the carboxy half (98, 99). These two main structural features of Vpr in the amino and carboxyl half are likely to interact with each other (99, 100). Early mutagenesis studies in mammalian cells suggested that the N-terminal α-helix-turn-α-helix is primarily responsible for nuclear localization of Vpr (85, 87, 90). The C-terminal end of Vpr, on the other hand, is responsible for the G2 arrest induced by Vpr (19, 85, 101). Consistent with the findings in mammalian cells, studies with a panel of Vpr mutations showed that the functional domains of G2 arrest and nuclear localization also mapped in fission yeast primarily to the C-terminal and the N-terminal halves of Vpr, respectively, (12). These structural requirements for G2 arrest and nuclear localization indicate that these two functions are independent both in mammalian and fission yeast cells (11, 12, 102).

The relationship between cell killing and other Vpr functions are less clear partly because there may be multiple mechanisms of cell killing by Vpr. For externally added peptides, the carboxyl half of Vpr was sufficient for killing (84). While this region is thought to be required for G2 arrest, studies in budding yeast localized this killing domain for externally added peptides to a region smaller than that required for G2 arrest (18, 103). Thus, this type of cell killing, presumably through MMP, does not require G2 arrest. However, studies in mammalian cells with expressed *vpr* have suggested that G2 arrest is required for killing by Vpr (5). Studies in fission yeast clearly indicate that G2 arrest is independent of cell killing based on studies with mutations in Vpr and Cdc2. Mutations in Vpr often had opposite effects on G2 arrest and cell killing in wild type fission yeast cells (12). One example is the F34I mutation which nearly eliminates cell killing but has no significant effect on the levels of G2 arrest. Further support for the independence of G2 and cell death comes from studies of the Y15F Cdc2 mutation in fission yeast (40). In the Y15F strain, where Vpr-induced G2 arrest is completely abolished, Vpr still induces cell killing.

One striking finding from the mutagenesis studies in fission yeast was that a single amino acid substitution at amino acid 33 (H33R) or 71 (H71R) could interrupt all three Vpr functions of G2 arrest, nuclear localization and cell killing (12). A point mutation E29G, which was isolated from a natural occurring virus, also affected all three activities of Vpr (Zhao et al., submitted). Interestingly, these single amino acid substitutions are present near the turn at amino acids 30-34 between the two amino terminal α helices or near the end of carboxy terminal α-helix (98, 99), suggesting that these regions maybe important in maintaining the overall tertiary structure required for all Vpr activities. These sites may represent useful targets in the future design of anti-Vpr therapies.

7. SUMMARY AND FUTURE PROSPECTS

Yeast is proving to be an excellent model system for studying Vpr activities. By in large, Vpr has very similar activities in yeast in comparison to human cells with respect to nuclear localization, cell cycle G2 arrest, morphological changes and cell death. Not only does Vpr have these activities in yeast, but there are additional similarities between the activities in yeast and human cells. For example, consistent with the findings in human cells, Vpr-induced G2 arrest correlates with inactivation of Cdc2 through increased inhibitory phosphorylation of Tyr15 on Cdc2. The regions of Vpr required for nuclear localization and G2 arrest are also quite similar between human and fission yeast cells. These numerous similarities justify studying the mechanisms of Vpr activities in yeast.

Work in yeast has thus far contributed to a number of important findings about Vpr activities. These include (1) Vpr induces cell cycle G2 arrest by going through a novel regulatory pathway, which includes a PP2A-mediated cellular mechanism, (2) Vpr plays its role in nuclear transport by binding to importin α at a previously unknown site, (3) MMP by Vpr is a major pathway for cell killing by Vpr, (4) Vpr disrupts the F-actin cytoskeleton which may be a second mechanism for cell killing by Vpr, (5) G2 arrest and cell death induced by Vpr are two independent functions in fission yeast and (6) a single amino acid substitution at E29G, H33R or H71R inacitvates three of the Vpr activities suggesting that these positions are important sites for maintaining the overall structure of Vpr. Given the powerful genetics technologies available in yeast, future work in yeast is likely to provide considerable additional insight into the molecular mechanisms of Vpr activities.

In addition to studies of mechanisms, yeast may also be useful in addressing the role of Vpr activities in viral infection and pathogenesis. Since Vpr displays multiple activities, a critical question is whether all of these activities are of equal importance to viral pathogenesis during the natural course of a HIV infection. Attempting to correlate Vpr activities with disease progression will require cloning the different *vpr* genes in a patient viral population and measuring their activities. Because of the simplicity of the yeast system and the similarity of Vpr activities in human and yeast cells, fission yeast provides an ideal system for this purpose. To this end, a new fission yeast expression vector system has been developed for rapid screening of Vpr activities (104). This rapid Vpr functional screening system provides an unique opportunity to directly address the relevance of Vpr functions to pathogenesis in HIV-infected patients. An early result from this screening system is that functionally defective *vpr* genes are frequently found in two HIV infected patients who did not progress to AIDS for over 10 years. In contrast, *vpr* genes from patients who rapidly progressed to AIDS were all fully active (Zhao et al., submitted). Thus, studies in yeast may provide insight not only into the mechanisms of Vpr activities but also into the role of these activities in viral infection and pathogenesis.

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