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Partner Molecules of Accessory Protein Vpr of the Human Immunodeficiency Virus Type 1

TOMOSHIGE KINO[1,2] and GEORGE N. PAVLAKIS[1]

ABSTRACT

Vpr (Viral protein-R) of the Human Immunodeficiency Virus type-1 is a 14-kDa virion-associated protein, conserved in HIV-1, -2 and the Simian Immunodeficiency Virus (SIV). Vpr is incorporated into the virion, travels to the nucleus, and has multiple activities including promoter activation, cell cycle arrest at the G2/M transition and apoptosis induction. Through these activities, Vpr is thought to influence not only viral replication but also numerous host cell functions. These functions may be categorized in three groups depending on the domains of Vpr that support them: (1) functions mediated by the amino terminal portion of Vpr, like virion packaging; (2) functions mediated by the carboxyl terminal portion such as cell cycle arrest; and (3) functions that depend on central α-helical structures such as transcriptional activation, apoptosis and subcellular shuttling. Association of these activities to specific regions of the Vpr molecule appears to correlate to the host/viral molecules that interact with corresponding portion of Vpr. They include Gag, host transcription factors/coactivators such as SP1, the glucocorticoid receptor, p300/CREB-binding protein and TFIIB, apoptotic adenine nucleotide translocator, cyclophilin A and 14-3-3 proteins. The properties of Vpr molecule has made it difficult to assess its function and determine the true cellular interactors. Further studies on Vpr function are needed to fully assess the function of this important early regulatory molecule of HIV and other lentiviruses.

INTRODUCTION

THE HUMAN IMMUNODEFICIENCY VIRUS (HIV) type-1 accessory protein Vpr (viral protein R) is a 96-amino-acid virion-associated protein shown to be important for virus replication/propagation *in vivo* (Connor *et al.*, 1995; Gibbs *et al.*, 1995; Goh *et al.*, 1998) (Fig. 1). It is a small basic protein conserved in HIV-1, -2 and the Simian Immunodeficiency Virus (SIV). Vpr is packaged in significant quantities into viral particles (Cohen *et al.*, 1990a; Paxton *et al.*, 1993), and is imported into the nucleus early after infection. These observations suggest that Vpr might play a role in early events in the viral life cycle (Hrimech *et al.*, 1999). Vpr has multiple activities such as reported participation in the nuclear translocation of the HIV-1–preintegration complex, transcriptional regulation of both the HIV-1 long terminal repeat and of cellular genes, cell-cycle arrest at the G_2/M boundary of cells, and possibly apoptosis (Popov *et al.*, 1998b; Heinzinger *et al.*, 1994; He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995; Rogel *et al.*, 1995; Cohen *et al.*, 1996; Emerman, 1996; Pavlakis, 1996; Ayyavoo *et al.*, 1997; Vodicka *et al.*, 1998; Stewart *et al.*, 1999). This information has been obtained by transfection experiments, and also in the context of whole viruses expressing or lacking Vpr. The reported functions of Vpr are obviously supported by its physical interactions with host/cellular molecules through specific sites on Vpr molecule. Many investigators have attempted the characterization of partner molecules binding to specific Vpr sites. The result has been the identification of many potential interactors. Some of them appear to be real, based also on functional data, but others may be false and may bind nonspecifically due to the extremely "sticky" nature of this viral protein. We review work on the identification of some Vpr interacting molecules and briefly discuss their relevance to the functions of this enigmatic small protein.

[1]Human Retrovirus Section, Center for Basic Research, National Cancer Institute-Frederick, Frederick, Maryland.
[2]Pediatric and Reproductive Endocrinology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland.

STRUCTURE OF Vpr

Several publications have proposed structures of Vpr fragments, whereas recently the whole Vpr structure was probed by nuclear magnetic resonance (NMR). The NMR structure of the amino-terminal half of a chemically synthesized Vpr molecule was first reported in 1999 (Wecker and Roques, 1999). In this analysis, Vpr (1–51) had a structure of "α-helix-turn-α-helix": Asp17–Glu29 forms the first α-helical structure with two faces of opposite amphipathicity, whereas Pro35–Glu46 contains the less amphipathic second α-helix. A β-turn (Ala30–His33) connects these two α-helices and leads to their quasi-perpendicular orientation (Fig. 2A). A recent publication analyzing a similar amino-terminal Vpr fragment indicates that proline residues in Vpr undergo *cis/trans* isomerization that may influence folding and the three-dimensional structure of Vpr (Bruns *et al.*, 2003). The structure of the chemically synthesized Vpr (52–96) has been also reported using a similar NMR analysis (Schuler *et al.*, 1999). A smaller fragment (amino acid 60–81) of Vpr has been called "LR (leucine–isoleucine-rich)" domain. It forms a long, well-defined amphiphilic α-helical structure extending from Trp53 to Arg78, which is consistent with a leucine-zipper motif (Fig. 2B). All of the hydrophobic amino acid side chains of this α-helix reside in one face of the helix, and thus form an extended uninterrupted hydrophobic surface, while polar residues mainly occupy the other side. A tail portion, which starts from amino acid 79, is called the "basic" region that contains seven arginine residues. This portion of Vpr is flexible, and does not form any strong secondary structures (Fig. 1B).

Recently, the three-dimensional NMR structure of the full-length Vpr showed the intact Vpr molecule also contains three α-helices, which fold around a hydrophobic core with flexible amino- and carboxyl-termini (Fig. 2C). The results also suggest that many of known mutations of Vpr introduced by reverse genetics may destroy this unique structure, indicating the limitations of mutational analyses for such a small molecule. Since Vpr forms complexes with several partner proteins, these might influence the reported structure. Crystallographic analysis of Vpr and of complexes with other molecules may provide more precise structural information in the future.

KNOWN Vpr FUNCTIONS AND INTERACTORS

Virion incorporation

Vpr is incorporated in the HIV-1 virion as multiple copies (~200) at a 1:7 ratio with capsid molecules (Cohen *et al.*, 1990a; Lu *et al.*, 1993; Muller *et al.*, 2000). As revealed by mutational analysis, the first α-helix (residues 17–29) in the amino terminal half of Vpr is responsible for incorporation of Vpr into the virion (Mahalingam *et al.*, 1995a, 1995b, 1997). Vpr incorporation is directed by its interaction with Gag protein (Table 1). Gag is produced as a p55 precursor molecule and is subsequently cleaved to form the matrix protein MAp17, the capsid protein CAp24, the nucleo-capsid protein NCp7 and the p6 protein, through enzymatic cleavages catalyzed by the viral protease. Carboxy-terminally located p6, but not the other fragments of Gag, interacts with Vpr. The presence of this sequence is sufficient for incorporation of Vpr into the virion as revealed by using several mutant HIV-1 clones that have chimeric *gag* genes with that of the Moloney Murine Leukemia Virus (MLV) (Kondo *et al.*, 1995). Amino acids 1–46 of p6, containing a leucine-triplet repeat sequence $(LXX)_4$, is responsible for the association with Vpr (Lu *et al.*, 1995). A subsequent report mapped the interaction domain in residues 32 to 46 of p6 that contains a LXXLF motif (Kondo and Gottlinger, 1996). Direct binding of Vpr and p6 is also confirmed in an *in vitro* binding assay employing bacterially produced and purified Vpr and p6 peptides (Jenkins *et al.*, 2001). The smallest Vpr fragment that supports the binding is amino acid 1–71, which contains amino-terminal α-helix that supports the virion incorporation of Vpr (Jenkins *et al.*, 2001). In addition to p6 gag, Vpr is also reported to associate with NCp7 and MAp17 (Jenkins *et al.*, 2001) (Table 1). In a later report, the NC domain of *Gag* is also shown to be required for virion incorporation of Vpr, using viral clones that have point mutations in this domain. Synthetic Vpr binds NCp7 *in vitro* at amino acids 52–96 of Vpr and zinc-finger domain of NCp7 (amino acids 13–51). Since an early report indicated that the carboxyl-terminal portion of Vpr (amino acid 84–94) also plays a role in virion incorporation of Vpr (Paxton *et al.*, 1993), NCp7 may cooperate with p6 gag for packaging of Vpr into virions (Roques *et al.*, 1997). Carboxyl-terminal portion of MAp17 seems to bind Vpr as well (Sato *et al.*, 1996). These results indicate that association of Vpr to p6 Gag appears to

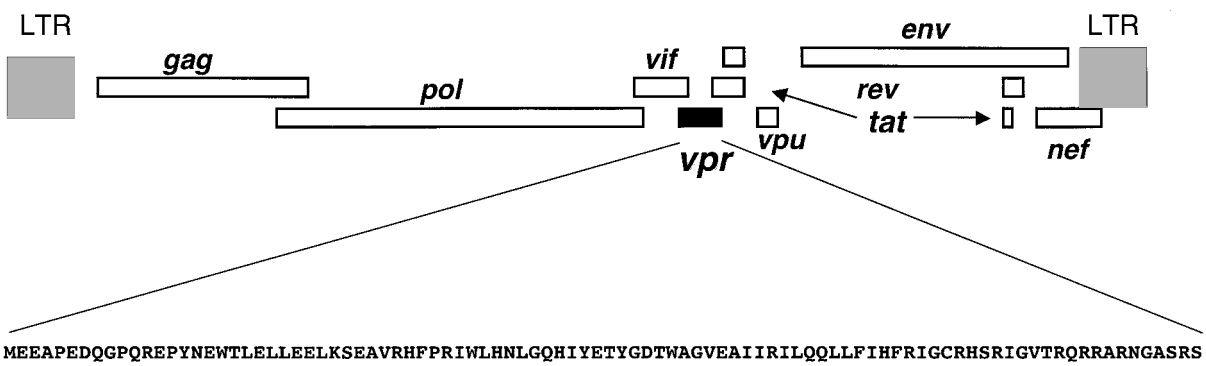

FIG. 1. HIV-1 proviral genome and the Vpr coding sequence.

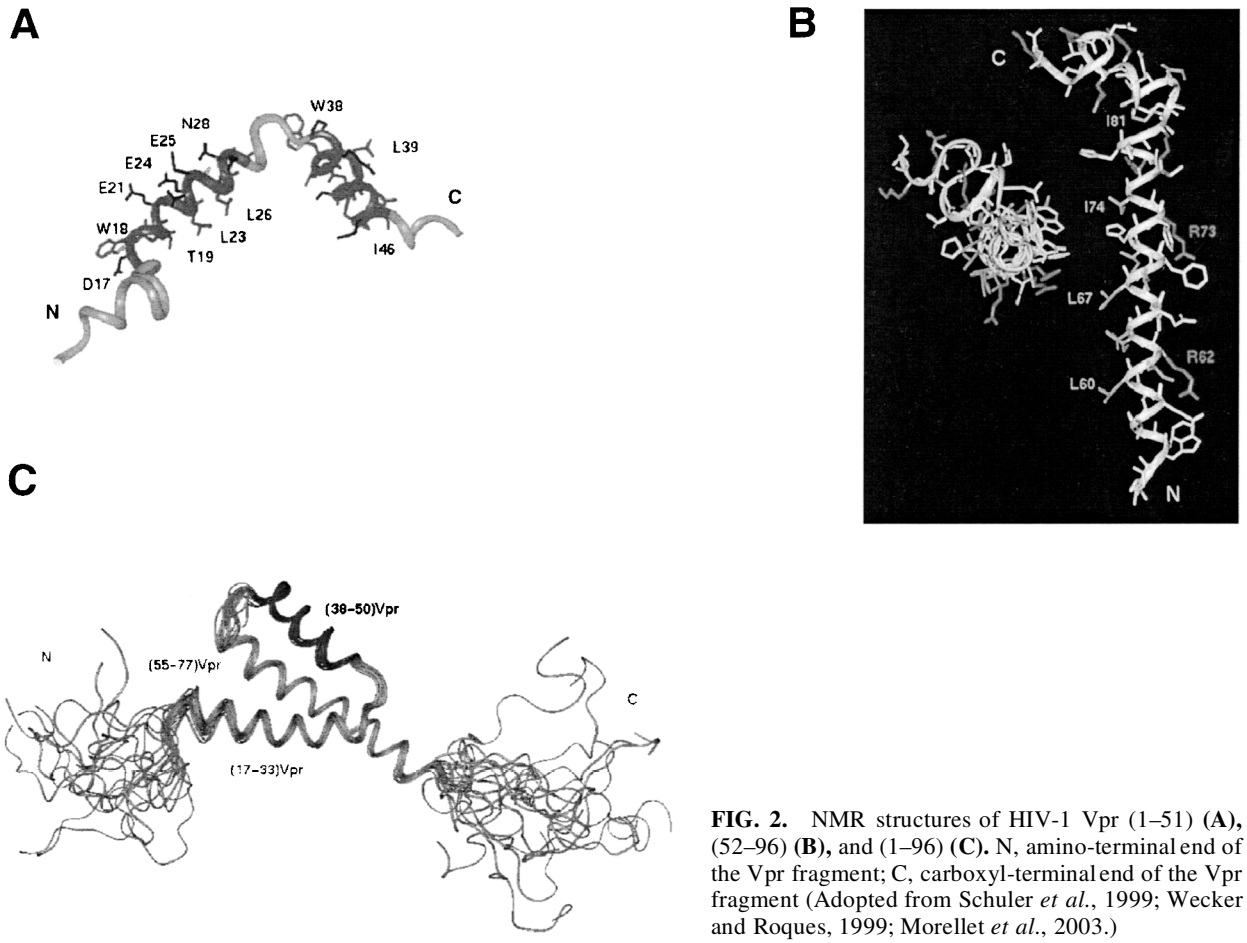

FIG. 2. NMR structures of HIV-1 Vpr (1–51) **(A),** (52–96) **(B),** and (1–96) **(C).** N, amino-terminal end of the Vpr fragment; C, carboxyl-terminal end of the Vpr fragment (Adopted from Schuler *et al.*, 1999; Wecker and Roques, 1999; Morellet *et al.*, 2003.)

play a major role in its incorporation into virions, while its interactions with NAp7 and MAp17 may play a supportive role based on mutational analysis using viral genome and actual virions. Inconsistency among the results may be due to different experimental conditions such as different viral strains and sensitivity of each employed assay system. Given that components of virions including p6 Gag, NCp7, MAp17, and viral RNA closely localize inside the virion, Vpr incorporation into virions may be initiated by p6 interaction and facilitated by multiple interactions with the Gag molecules though its multiple surfaces spanning almost the entire molecule (Mahalingam *et al.*, 1995c).

Nuclear localization and trafficking of the provirus into the nucleus

For most retroviruses, the newly synthesized viral DNA within the preintegration complex gains access to the nucleus of the infected cells after the dissolution of the nuclear membrane during mitosis. In contrast, HIV-1 and the other lentiviruses can integrate into the host DNA by transporting the preintegration complex (PIC) into the nucleus even in nondividing cells. Vpr and MAp17 are nucleophilic components that have been proposed to participate in nuclear localization of viral nucleic acids in nondividing cells. These two components may provide functional redundancy. Nuclear translocation of the preintegration complex was found to be energy dependent; therefore, the active nuclear transport mechanism through the nuclear pore/importin system was suggested to mediate the process. Basic, functional nuclear localization signals (NLS) were found in MAp17, but not in Vpr (Haffar *et al.*, 2000). Mutational analyses revealed that the first and second α-helical structures of Vpr both act on the nuclear translocation of PIC as well as nuclear localization of Vpr (Di Marzio *et al.*, 1995; Yao *et al.*, 1995; Mahalingam *et al.*, 1997; Nie *et al.*, 1998). The third α-helix of Vpr may also influence the nuclear localization of Vpr using several Vpr mutants and green fluorescent protein chimeras (Sherman *et al.*, 2001). Subsequently, Vpr was found to bind importin α, possibly via its first amino-terminal α-helix in an *in vitro* binding assay using bacterially produced purified proteins (Table 1). It was proposed that Vpr facilitates import of PIC via several postulated mechanisms (Popov *et al.*, 1998a, 1998b; Vodicka *et al.*, 1998). Direct association of Vpr to nucleoporin was also shown in the same binding assay (Popov *et al.*, 1998a; Vodicka *et al.*, 1998).

In addition to nuclear translocation, Vpr may also be exported from the nucleus into the cytoplasm, possibly shuttling between these two components. The nuclear export of Vpr appears to be catalyzed by exportin-1/chromosome maintenance region 1 protein (CRMI)-dependent classic nuclear export path-

TABLE 1. VPR INTERACTING PROTEINS

Interactors	*Portion of the protein supporting the binding*	*Portion of Vpr supporting the binding*	*Biological significance of the interaction*	*References*
HIV-1 proteins/components				
NCp7 (Gag)	The zinc-finger domain The leucine-rich motif "LXSLFG"	Carboxyl-terminal portion (amino acid 80–96)	Incorporation of Vpr into HIV-1 virion	(de Rocquigny *et al.*, 1997; Li *et al.*, 1996)
P6 (Gag)		Amino-terminal α-helical structure	Incorporation of Vpr into HIV-1 virion	(Bachand *et al.*, 1999; Kondo and Gottlinger, 1996; Lu *et al.*, 1995)
MA p17	Not determined	Not determined	Incorporation of Vpr into HIV-1 virion	(Sato *et al.*, 1996)
Vpr	Amino terminal portion (amino acid 26–42)	Amino-terminal portion (amino acid 26–42)	Oligomerization	(Zhao *et al.*, 1994a)
Vpr	Carboxyl-terminal half (amino acid 52–96)	Carboxyl-terminal half (amino acid 52–96)	Homodimerization	(Schuler et al., 1999)
Tat	Not determined	Not determined	Enhancement of Tat-transactivation of HIV-1-LTR	(Sawaya *et al.*, 2000)
HIV-1 RNA	1–415 bps including entire LTR	Carboxyl-terminal half (amino acid 52–96)	Incorporation of Vpr into HIV-1 virion?	(Schuler *et al.*, 1999)
Cellular proteins/components				
R-interacting protein (RIP)	Not determined	Amino-terminal LR domain	Nuclear translocation	(Zhao *et al.*, 1994b)
Transcription factor IIB (TFIIB)	Amino-terminal portion (amino acid 1–111)	Amino acid 15–77	Transactivation of promoters	(Agostini *et al.*, 1996)
Cyclin T1	Amino acid 300–479	Not determined	Enhancement of Tat-transactivation of HIV-1-LTR	(Sawaya *et al.*, 2000)
SP-1	Not determined	Central Leu/Ile-rich domain	Transactivation of the HIV-1-LTR	(L. Wang *et al.*, 1995)
Proteasomal subunit mov34 homolog (hVIP/mov34)	Carboxy-terminal portion	Not determined	Cell-cycle arrest at G_2/M phase? Glucocorticoid receptor-mediated actions	(Mahalingam *et al.*, 1998)
Uracil DNA glycosylase	WXXF motif	Amino acid 15–77	Mutation rate of the HIV-1 genome	(Bouhamdan *et al.*, 1996; Mansky *et al.*, 2000; Selig *et al.*, 1997)

HHR23A	UBA (2) domain	Amino acid 25–77	Cell-cycle arrest at G_2/M phase and apoptosis?	(Dieckmann *et al.*, 1998; Gragerov *et al.*, 1998; Mansky *et al.*, 2001; Withers-Ward *et al.*, 1997)
Importin α	Not determined	Amino-terminal portion (F34)	Nuclear translocation of the HIV-1 preintegration complexes and nuclear localization of Vpr	(Popov *et al.*, 1998b; Vodicka *et al.*, 1998)
Nucleoportin	Not determined	Not determined	Nuclear translocation of the HIV-1 preintegration complexes and nuclear localization of Vpr	(Popov *et al.*, 1998a; Vodicka *et al.*, 1998)
Cellular DNA	Not determined	Carboxyl-terminal portion	Nuclear localization of Vpr	(Zhang *et al.*, 1998)
The glucocorticoid receptor	Ligand-binding domain	LXXLL motif (amino acid 6468)	Transactivation of the glucocorticoid-responsive promoters	(Kino *et al.*, 1999)
Adenine nucleotide translocator (ANT)	Not determined	Carboxyl-terminal portion (Amino acids 52–96)	apoptosis	(Jacotet *et al.*, 2000, 2001)
p300/CREB-binding protein	Amino acid 2045–2191	Amino acids 66–82	Transactivation of the HIV-1-LTR and glucocorticoid-responsive promoters	(Kino *et al.*, 2002)
Lys-tRNA synthetase	Not determined	Amino terminal half	HIV-1 reverse transcription	(Stark and Hay, 1998)
cyclophilin A	Not determined	Amino terminal portion	*de novo* synthesis of Vpr	(Zander *et al.*, 2003)
14-3-3- proteins	The 8^{th} α-helix	Carboxyl-terminal portion	Cell cycle arrest at G_2/M phase	(Kino *et al.*, 2004)

way. Nuclear export activity of Vpr is mapped to the third α-helix by mutational analysis (Sherman *et al.*, 2001). Since a putative nuclear export signal (NES) is not found in Vpr molecule, Vpr may associate with a cellular molecule that has NES.

Promoter activation

Vpr was initially reported as an activator of the HIV-1 long terminal repeat (LTR) and several heterologous viral promoters (Cohen *et al.*, 1996b). This observation indicates that Vpr packed into virions may stimulate transcription of the viral mRNA in the very early stage of infection, prior to Tat production. In an early study consistent with this hypothesis, Vpr was proposed to stimulate the HIV-1 long terminal repeat (LTR) promoter by associating with the transcription factor SP1 (Table 1). Vpr bound to SP1 in a gel-shift assay using probes containing the SP1-responsive elements and in a coimmunoprecipitation assay (L. Wang *et al.*, 1995). Direct binding of the two molecules was not demonstrated, but the second α-helix of Vpr is necessary for stimulation of the HIV-1-LTR. Vpr was also shown to interact with one of the general transcription factor (TF) TFIIB in an *in vitro* (Table 1) GST-pull down assay (Agostini *et al.*, 1996) (Table 1). Subsequent analyses further indicated that Vpr functions as a potent enhancer of Tat-induced activation of the HIV-1-LTR (Forget *et al.*, 1998; Kino *et al.*, 2002). Interactions of Vpr with Tat and its partner protein CyclinT1 were also shown *in vitro* (Table 1), but their biological significance is uncertain since both molecules are extremely sticky (Sawaya *et al.*, 2000) (Table 1). This is actually a persistent problem of all Vpr binding studies, requiring the implementation of additional methods to verify relevance of the identified interactions. We recently investigated additional potential Vpr interactions and found that Vpr acts as an adaptor molecule bridging promoter-bound transcription factors and the transcriptional coactivator p300/cAMP-responsive element-binding protein (CREB)-binding protein (CBP) (Kino *et al.*, 2002) (Table 1). This family of large coactivator molecules binds to numerous transcription factors and several viral molecules including adenovirus E1A and HIV-1 Tat, functioning as a signal integrator of numerous cell activities at the level of transcription (Goodman and Smolik, 2000). Vpr, via its third α-helix, binds directly to amino acid 2045–2191 of human p300, a region also known to associate with host coactivator p160 family proteins (McKenna *et al.*, 1999; Kino *et al.*, 2002). The binding of the two molecules is confirmed in several available binding assays both *in vitro* and *in vivo.*

Vpr was proposed to interact with the glucocorticoid receptor via cellular 41-kDa protein (Refaeli *et al.*, 1995; Goodman and Smolik, 2000). We examined the Vpr–glucocorticoid receptor association and identified that Vpr binds directly to the glucocorticoid receptor via its conserved LXXLL motif located at amino acids 64 to 68, and markedly potentiates glucocorticoid receptor action on its responsive promoters, acting as a nuclear receptor coactivator (Kino *et al.*, 1999) (Table 1). We showed that Vpr is a general coregulator of nuclear receptors that influences not only the glucocorticoid receptor but also the progesterone and estrogen receptors (Kino and Chrousos, 2003).

Cell cycle arrest at the G2/M transition

Vpr arrests HIV-1–infected cells at the G2/M boundary of the cell cycle (He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995). Through this activity of Vpr, it has been proposed to facilitate viral propagation (Goh *et al.*, 1998). This activity of Vpr is mapped in its carboxyl-terminal basic portion using mutational analysis (Di Marzio *et al.*, 1995). Transition through the G_2/M check point in mammalian cells is strictly controlled by activation of a protein complex formed by a catalytic subunit, the cyclin-dependent kinase Cdc2, and its regulatory partner CyclinB1, through coordinated phosphorylation/dephosphorylation events (Jackman and Pines, 1997; Ohi and Gould, 1999) (Fig. 4). The protein kinases Wee1 and Myt1 inactivate this complex by phosphorylating threonine residues at amino acids 14 and 15 of Cdc2, while the phosphatase Cdc25C activates it by dephosphorylating the same threonine residues (Jackman and Pines, 1997; Ohi and Gould, 1999; Nebreda and Ferby, 2000). Threonine, at amino acid 161 of Cdc2, is phosphorylated by Cdk-activating kinases. Upstream kinases such as Cdk2, which is stimulated by several signals such as DNA damage, stimulates Wee1 but suppresses Cdc25C by phosphorylating serine residues at amino acid 549 of Wee1 and 216 of Cdc25C, respectively, and induces cell-cycle arrest at G_2/M phase. Protein phosphatase 2A dephosphorylates serine residue at amino acid 549 of Wee1 and inactivates this kinase (Fig. 3). Stimulated Cdc2/CyclinB1 complexes then phosphorylate Wee1 and Cdc25C, creating a positive feedback loop.

In this cascade, it is known that Vpr inactivates the Cdc2/CyclinB1 complex by keeping Cdc2 at a hyperphosphorylated state (He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995; Rogel *et al.*, 1995; Elder *et al.*, 2000; Masuda *et al.*, 2000). This is possible by modulating the function of host protein(s), which act upstream of Cdc2/CyclinB1, such as PP2A, Wee1, Myt1, and Cdc25C. Vpr is reported to modulate activity and/or protein levels of PP2A and Wee1 with yet unknown mechanisms (Tung *et al.*, 1997; Elder *et al.*, 2001; Yuan *et al.*, 2003). Vpr expression also causes cell-cycle arrest in fission yeast. Using genetic analysis, the cell-cycle arresting activity of Vpr was associated with presence of *pp2a*, *wee1*, and *rad24* (Masuda *et al.*, 2000). *rad24* encodes protein with homology to the 14-3-3 family of proteins in humans (Rosenquist *et al.*, 2000).

Extensive searches have been undertaken to identify host molecules that specifically support the cell-cycle arresting activity of Vpr. Using an yeast two-hybrid screening assay, the human homolog of yeast mov34 (hVIP/mov34), a kinase, was identified as Vpr interactor (Table 1). hVIP/mov34 belongs to a family of transcriptional regulators and proteasomal members, and acts to overcome the G_2/M transition of the cell cycle (Maghalingam *et al.*, 1998). Binding of Vpr to hVIP/mov34 is mediated though carboxyl-terminal portion of the latter in an *in vitro* binding assay, but evidence indicating involvement of this protein to Vpr cell cycle arrest is not shown yet (Mahalingam *et al.*, 1998; Ramanthan *et al.*, 2002). By yeast two-hybrid screening assays, two groups have found that HHR23A, a human homolog of yeast rad23, interacts with Vpr through the carboxyl-terminal portion containing the UBA domain of the latter molecule (Withers-Ward *et al.*, 1997, 2000; Dieckmann *et al.*, 1998; Gragerov *et al.*, 1998) (Table 1). Interesting, exogenous expression of HHR23A suppressed the cell-cycle arrest of Vpr in our hands (Gragerov *et al.*, 1998). Recent mutational analysis by another group did not find correlation of binding and the cell cycle arrest activity (Mansky *et al.*, 2001). These results may indicate that HHR23A is not mediating the cell-cycle arrest effects of Vpr.

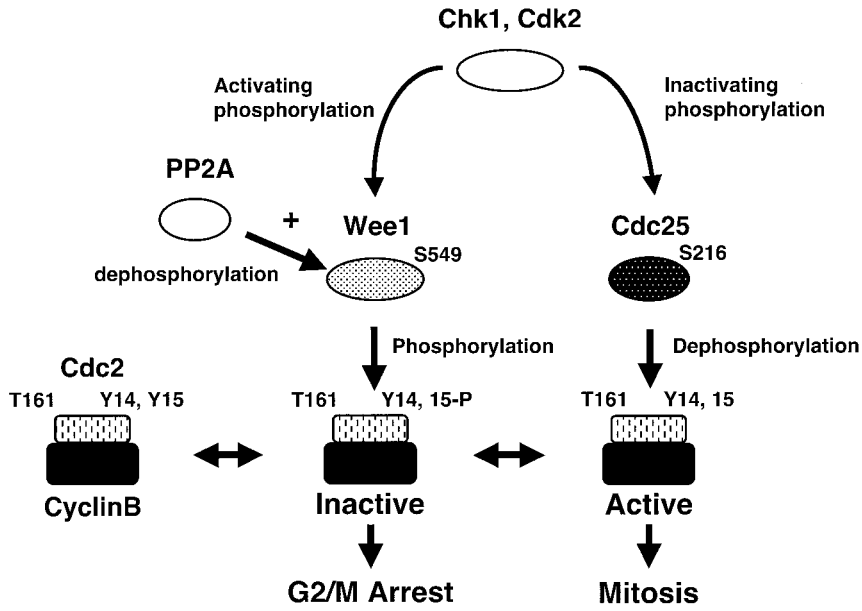

FIG. 3. Regulatory mechanisms of the G_2/M transition of the cell cycle by cellular kinases and phosphatases.

We have recently performed extensive yeast two-hybrid screening assays using a panel of wild-type and mutant Vpr molecules. We first repeated the screening with wild-type Vpr as a bait and found several potential Vpr-interacting molecules (Table 2). These include several interesting proteins that might have relevance for known Vpr functions, in addition to reported Vpr-associating proteins such as importin α and HHR23A. For example, IκB and the signal transducer and activator of transcription 5 B (STAT5B) may participate in Vpr-induced modulation of the immune function (Ayyavoo *et al.*, 1997; Roux *et al.*, 2000; Muthumani *et al.*, 2002a). C-terminal binding protein 2 (CtBP2), retinoblastoma-binding protein (RbAp46) and RNA polymerase II subunit hsRBP7 may participate in Vpr's transcriptional activity. In parallel experiments, we assessed binding of potential interactors to the mutant VprL64A, which has cell-cycle arrest activity but does not bind to the glucocorticoid receptor (Kino *et al.*, 1999). All of potential interactors found in this screening, bound VprR80A but not VprL64A mutant, which indicates they do not have cell-cycle arresting activity (Kino *et al.*, 1999).

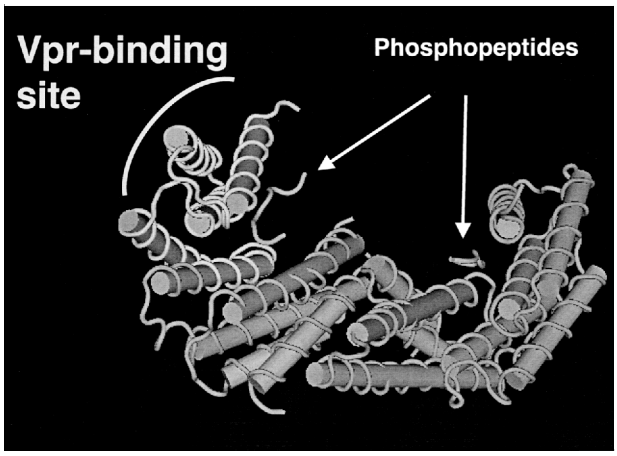

FIG. 4. The Vpr binds to the C-terminal portion of 14-3-3. The Vpr binding site is shown in the 14-3-3ξ molecule obtained by the crystallographic analysis. (Modified from Yaffe *et al.*, 1997.)

Since we did not identify any molecules that can interact with VprL64A mutant, which has wild-type–equivalent cell-cycle arresting property, we performed a yeast two-hybrid screening using this mutant as a bait (Kino *et al.*, 2004) (Table 3). This approach was very promising, because it gave much fewer hits in this assay, eliminating a large number of cellular proteins that bind to wild-type Vpr. Interestingly, we found several human 14-3-3 proteins, which play a central role in the cell-cycle regulation, bind wild-type as well as this mutant Vpr (Tables 1 and 3). We subsequently found that 14-3-3 proteins increase Vpr-induced cell cycle arrest. Lack of one of the 14-3-3 proteins (14-3-3σ) significantly reduced Vpr cell-cycle arrest. These results indicate that 14-3-3 contributes to the cell-cycle arresting activity of Vpr.

14-3-3 protein family consists of nine isotypes produced from at least seven distinct genes in vertebrates. 14-3-3 proteins bind phosphorylated serine/threonine residues at specific positions of their partner proteins and regulate their activities by changing their subcellular localization and/or stability. 14-3-3 contain nine α-helical structures and form homo- and hetero-dimers through their amino-terminal portion (Yaffe *et al.*, 1997; Rittinger *et al.*, 1999; Fu *et al.*, 2000; Muslin and Xing, 2000; Rosenquist *et al.*, 2000) (Fig. 4). The central third to fifth α-helices create a binding pocket for a phosphorylated serine/threonine residue and the carboxyl-terminal seventh to ninth helices determine the specificity to target peptide motifs (Yaffe *et al.*, 1997; Rittinger *et al.*, 1999). 14-3-3 contains a nuclear export signal (NES) in the ninth helix (Rittinger *et al.*, 1999; Kino *et al.*, 2003).

TABLE 2. SUMMARY OF THE YEAST TWO-HYBRID SCREENING USING WILD-TYPE VPR AS BAIT (TOTAL CLONES SEQUENCED: 159)

Potential interactors	*Genbank accession number*	*Number of clones*	*Interaction with Vprs*			
			WT	*L64A*	*R80A*	*64–96*
D123	D14878	5	+	−	+	+
HHR23A	D21235	4	+	−	+	−
Rch1 (Importin α)	U09559	3	+	−	+	+
Tre oncogene	X63546	3	+	−	+	+
Cullin 1	AF062536	2	+	−	+	+
ATP-binding protein	AJ01084	1	+	−	+	−
Chromosome-associated polypeptide (HCAP)	AF020043	1	+	−	+	−
C-terminal binding protein 2 (CtBP2)	AF016507	1	+	−	+	−
MAD-3 (I-κB)	M69043	1	+	−	+	+
PDCD (programmed cell death-2/Rp8 homolog)	S78085	1	+	−	+	−
Putative SMC-like protein	AJ005015	1	+	−	+	−
Retinoblastoma-binding protein (RbAp46)	U35143	1	+	−	+	−
RNA polymerase II subunit hsRBP7	U20659	1	+	−	+	−
Signal transducer and activator of transcription 5B (STAT5B)	U47686	1	+	−	+	+
TOM-1-like protein	AJ010071	1	+	−	+	−

14-3-3 proteins play a significant role in cell-cycle progression at several different stages (Fig. 5). First, they regulate Cdc25C activity (Peng *et al.*, 1997; Sanchez *et al.*, 1997; Zeng *et al.*, 1998; Dalal *et al.*, 1999; Lopez-Girona *et al.*, 1999; Rittinger *et al.*, 1999; Morris *et al.*, 2000; Graves *et al.*, 2001). Second, they bind Wee1 kinase and increase the stability and activity of this protein (Y. Wang *et al.*, 2000; Lee *et al.*, 2001). Third, 14-3-3 also bind and activate Chk1 and Cdk2 kinases,

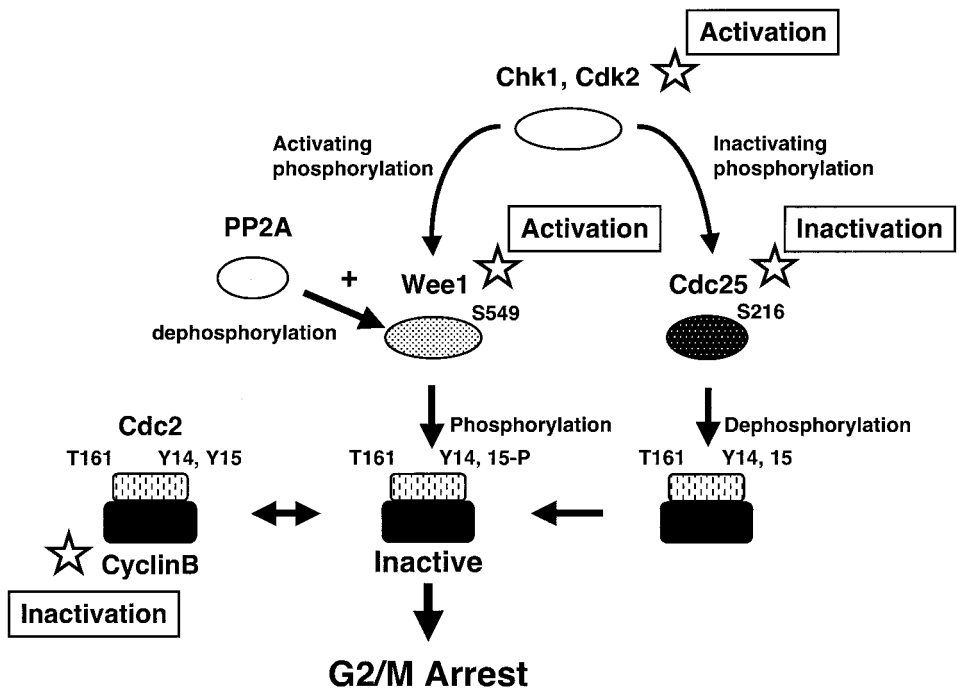

FIG. 5. 14-3-3 Participates in the regulation of the G_2/M transition of the cell cycle at several different steps. Known affected sites are indicated with stars *. (Based on the results in Peng *et al.*, 1997; Chan *et al.*, 1999; Chen *et al.*, 1999; Y. Wang *et al.*, 2000.)

by appropriately sequestering these molecules inside the nucleus (Chen *et al*., 1999). Activation of Chk1 causes phosphorylation of Cdc25C, producing a binding site for 14-3-3 proteins, leading to inactivation of the phosphatase (Sanchez *et al*., 1997; Zeng *et al*., 1998). Fourth, 14-3-3 proteins bind both phosphorylated Cdc2 and CyclinB1, and inactivate the complex by exporting it into the cytoplasm (Chan *et al*., 1999; Laronga *et al*., 2000). In our analyses, we examined Vpr on the Cdc25C localization to test the functional relevance of Vpr binding to effector molecules of 14-3-3, and found that Vpr induces cytoplasmic translocation of Cdc25CS216A mutant, which cannot bind 14-3-3 and thus regularly stays in the nucleus (Kino *et al*., 2004). Since Vpr binds 14-3-3 proteins at their carboxyl-terminal part, a region that plays an important role in its binding specificity to phosphopeptides (Rittinger *et al*., 1999), binding of Vpr may alter the binding affinity of 14-3-3 to its partner proteins. Alternatively, Vpr might facilitate or inhibit the association of 14-3-3 proteins to their partner molecules with additional interactions. Therefore, it is highly possible that Vpr also modulates activities of Wee1, Chk1, Cdk2, and, possibly, Cdc2/CyclinB1 complex by changing their binding specificity to 14-3-3 proteins. Indeed, a recent report indicates that Vpr downregulates protein levels of Wee1 (Yuan *et al*., 2003). Since 14-3-3 increases stability of this kinase (Lee *et al*., 2001), the reported Vpr effect may be the result of 14-3-3/Wee1 association.

Apoptosis induction

The significant depletion of the CD4(+) T cells is one of the landmarks of acquired immunodeficiency syndrome (AIDS) in HIV-1–infected patients. Vpr may be one of the viral factors that may cause decrease of the T cell population by inducing apoptosis. It was suggested recently that VprR77Q, that is incapable of inducing apoptosis, is associated with long-term nonprogressive HIV-1 infection, indicating that apoptotic activity may have relevance to viral pathogenecity (Lum *et al*., 2003). Vpr can induce apoptosis not only in peripheral lymphocytes but also in several tumor cell lines and in neuronal cells (Stewart *et al*., 1997, 1999; Patel *et al*., 2000). The Vpr domain responsible for this activity is mapped within amino acids 1–70 of Vpr, which does not contain the domain responsible for cell-cycle arrest (Nishizawa *et al*., 2000; Jian and Zhao, 2003), indicating that apoptosis and cell-cycle arresting activity are two separate functions. Vpr induces apoptosis by activating cysteinyl aspartate-specific proteases (caspases) (Stewart *et al*., 2000; Muthumani *et al*., 2002b), possibly by its direct effect on mitochondria (Jacotot *et al*., 2000). Vpr may increase the permeabilization through the permeability transition pore complex (PTPC). Carboxyl-terminal half of Vpr is associated with the PTPC component adenine nucleotide translocator (ANT) *in vitro* (Table 1). Bcl-2, a known interactor of ANT, reduces the ANT-Vpr interaction, as determined by affinity purification and plasmon resonance studies (Jacotot *et al*., 2000, 2001).

Other Vpr functions and potential interactors

Vpr is reported to interact with one of the DNA repair enzymes, uracil DNA glycosylase (UNG) (Table 1). Their interaction was originally found in a GAL4-based yeast two-hybrid screening and their interaction domains are mapped in 15–77 of Vpr and WXXF motif of UNG, respectively. The interaction may be relevant to the incorporation of UNG into HIV-1 virions. Presence of Vpr and UNG in the viral particle is correlated with reduction of mutation rates of the HIV-1 genome that is possibly catalyzed by the incorporated UNG (Bouhamdan *et al*., 1996, 1998; Selig *et al*., 1997).

Using a LexA-based yeast two-hybrid system, Vpr was shown to interact with Lys–tRNA synthetase (Stark and Hay, 1998) (Table 1). Binding was mapped within amino-terminal half of Vpr *in vitro* and suppressed activity of this enzyme. The results suggest that Vpr may influence the initiation of HIV-1 reverse transcription, since the $tRNA^{LYS}$ synthesized by this enzyme functions as a primer of reverse transcription.

Recently, Vpr was found to interact with cyclophilin A, a host peptidylprolyl *cis/trans* isomerase (PPIase) that is also the receptor for cyclosporine and is incorporated in HIV-1 virions (Zander *et al*., 2003) (Table 1). The amino-terminal portion of Vpr including proline at amino acid 35 is required for this association *in vitro*. It was proposed that this interaction may be important for the *de novo* synthesis of Vpr.

Vpr is hypothesized to form an oligomer or dimmer from its physical characteristics. Through its aggregative property, it forms an ion channel on the artificial lipid bilayer membrane (Piller *et al*., 1998, 1999) (Table 1). The channel formed by Vpr causes a large inward current, and can cause death in cultured neuronal cells. Since Vpr is found in the cerebrospinal fluid of HIV-1–infected patients who develop dementia (Levy *et al*., 1994; Albright *et al*., 2003), Vpr might be involved in the development of this pathologic condition through this activity.

TABLE 3. SUMMARY OF THE YEAST TWO-HYBRID SCREENING USING VPRL64A AS BAIT (TOTAL CLONES SEQUENCED: 96)

Potential interactors	*GenBank accession number*	*Number of clones*	*Interaction with Vpr:*		
			WT	*L64A*	*R80A*
DNA-PK interacting protein (KIP)	U85611	15	+	+	±
Cyclin kinase inhibitor p27	U10906	8	+	+	−
Huntington-interacting protein 2 (HIP)	U58557	5	+	+	−
14-3-3η	X78138	5	+	+	−
14-3-3θ	X56468	1	+	+	−
14-3-3τ	D87662	1	+	+	−

Finally, Vpr was found to interact directly to nucleic acids such as host DNA and HIV-1 RNA (Zhang *et al.*, 1989; Zander *et al.*, 2003) (Table 1). Binding of Vpr to DNA is supported by its carboxyl basic portion, and the interaction may be relevant also for transcriptional activity of Vpr. Vpr binds 1–415 of HIV-1 RNA that contains entire LTR, and their interaction may occur in HIV-1 virion.

SUMMARY

Despite its small size, Vpr has multiple functions that influence not only viral replication/proliferation but also numerous host cell activities. These functions may be categorized to three groups depending on the domains of Vpr that support them: (1) functions that is mediated by the amino-terminal portion of Vpr, like virion packaging. (2) Activities conducted by the carboxyl-terminal portion such as the cell-cycle arresting activity. (3) Functions that depend on central α-helical structures such as transcriptional activation, apoptosis, and subcellular shuttling. Distribution of these activities inside the Vpr molecule appear to correlate to the host/viral molecules that specifically interact with corresponding portion of Vpr.

Assessing Vpr function and interactions face significant problems caused by the nature of this viral protein, that is, instability including aggregation tendency. Since Vpr is a small molecule, disruption of any specific domain by insertion of mutations frequently causes major structural changes, and affects the rest of the molecule. It is also a very sticky molecule, and as a result, it interacts with a big number of proteins. Some Vpr functions appear to be supported by interaction with multiple molecules. These factors significantly complicate functional analysis, and may cause inconsistent results from different laboratories that employ different assay systems. However, development of Vpr characteristics appears to be an integral part of the strategy of HIV-1 to increase the chances of its replication.

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Address reprint requests to:
George N. Pavlakis, M.D., Ph.D.
Human Retrovirus Section
Center for Cancer Research
National Cancer Institute–Frederick
Bldg. 535, Rm. 210
Frederick, MD 21702-1201

E-mail: pavlakis@ncifcrf.gov