Pro-apoptotic Activity of HIV-1 Auxiliary Regulatory Protein Vpr Is Subtype-dependent and Potently Enhanced by Nonconservative Changes of the Leucine Residue at Position 64*

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Destruction of CD4+ T cells, the hallmark of AIDS, is caused in part by HIV-1-induced apoptosis of both infected cells and noninfected “bystander” cells. The HIV-1 auxiliary regulatory protein Vpr has been shown to harbor a pro-apoptotic activity that may contribute to cellular and tissue damage during AIDS pathogenesis. The biochemical mechanism of this Vpr function remains unclear. In this report, substitutions of a single amino acid residue Leu64 with Pro, Ala, or Arg are shown to dramatically enhance the pro-apoptotic activity of Vpr, as evidenced by the degradation of cellular DNA into fragments of 200-bp increments. Substitutions of Leu64 with conservative residues have no effect. The pro-apoptotic activity of the VprL64P mutant also requires activation of caspase(s) and is inhibited by the secondary mutation I61A, indicating a high specificity for Vpr-induced apoptosis. Among the three HIV-1 subtypes examined, a subtype B Vpr and an A/G subtype recombinant Vpr have a moderate level of pro-apoptotic activity, whereas a subtype D Vpr has no detectable activity. However, the L64P mutation efficiency enhances the pro-apoptotic potential of the subtype B and subtype D Vpr molecules but not that of the A/G recombinant Vpr. It is hypothesized that Vpr molecules from different HIV-1 subtypes as well as Vpr variants that emerge during HIV-1 infection may have different pro-apoptotic potentials and contribute to the diversity of AIDS pathogenesis.

HIV-1 infection leads to progressive depletion of CD4+ T cells and immune deficiency. This is partly because of the direct cytopathic effects of HIV-1 replication in the CD4+ host cells and enhanced apoptosis of uninfected “bystander” cells (1, 2). Examination of lymph node biopsies has revealed occurrence of apoptosis predominantly in uninfected cells that are in close vicinity of infected cells (3, 4). In the brain of AIDS patients, apoptosis of neurons is also prevalent despite the absence of direct neuronal infection by HIV-1 (5, 6). This pattern of apoptosis during clinical HIV-1 infection appears to be because of the ability of HIV-1 to circumvent apoptosis of infected cells, at least at the early phase of the viral life cycle, for the benefit of viral replication. Enhanced apoptosis of “bystander” cells during HIV-1 infection may therefore be a result of interactions between the host cells and soluble viral and immunological factors (4).

Different HIV-1 proteins interact with the cellular regulatory pathways for apoptosis and have a direct impact on the survival of both the infected and uninfected cells. The envelope gp120 has been shown to interact with the chemokine receptor CXCR4 and cause apoptosis of CD4+ or CD8+ T cells, as well as neurons (7, 8). HIV-1 Nef has been found to have both pro- and anti-apoptotic activities. Nef produced during HIV replication enhances expression of the Fas ligand (FasL) on the surface of infected cells, causing apoptosis of bystander cells through FasL-Fas cross-linking (9). On the other hand, Nef protects the infected cells from apoptosis by inhibition of the apoptosis signal-regulating kinase 1 (ASK1) or inhibition of the pro-apoptotic protein Bad through increased phosphorylation by the p21-activated kinase (10, 11).

HIV-1 Vpr is a 96-amino acid, 14-kDa auxiliary regulatory protein with pro-apoptotic activities in many cell types (12–17). Vpr-transgenic mice have thymus atrophy, apoptosis of thymocytes, and a dramatic decrease of T cell numbers in lymph nodes and in circulation (15). In groups of so-called “long-term nonprogressors,” mutations or truncations of Vpr have been identified that reduce or abolish the pro-apoptotic potential of Vpr (16, 18). Based on these results, it has been proposed that the pro-apoptotic activity of Vpr may contribute to the pathogenesis of AIDS (16, 18). In this report, mutational analysis shows that a single L64P, L64A, or L64R mutation dramatically enhances the pro-apoptotic potential of Vpr. The results also suggest that the pro-apoptotic activity of Vpr is dependent on the HIV-1 subtype and is affected significantly by genetic recombination. These results may lead to a better understanding of the contribution of Vpr to AIDS pathogenesis.

EXPERIMENTAL PROCEDURES

DNA Constructs—For most of the constructs, the HIV-1 isolate 89.6 (GenBank™ accession number U39362) was used as the template for PCR cloning. The pFSZ3 vector was modified from pFSZ2 (19). Three DNA fragments were ligated first to generate an intermediate vector pFSZ3(G-): the XhoI/XbaI fragment from pFSZ2 as the vector backbone, the XhoI/PstI digested p89.6/PCR1 DNA (with primers P1/P2), and the PstI/XbaI digested p89.6/PCR2 DNA (with primers P3/P4). Subsequently, PstI-digested GFP-PCR DNA (with primers P5/P6) was inserted into the PstI site of pFSZ3(G-) to generate pFSZ3. The wild type construct pFSZ3-Vpr was generated by substituting the Stul/EcoRI fragment of pFSZ3 with the Stul/EcoRI fragment of pFSZ2-Vpr(19). pFSZ3-TAA is modified from pFSZ3-Vpr to carry a premature stop codon in place of the fifth codon in the Vpr ORF. Except as described otherwise, all Vpr mutants were generated by cloning of either a BamHI/EcoRI-digested PCR DNA or a BamHI/SalI-digested PCR DNA into the corresponding sites of pFSZ3.
**Vpr Apoptotic Activity Determined by Leu^64 and Viral Subtype**

pFSZ3-L, pFSZ3-R, pFSZ3-D, pFSZ3-DIP, and pFSZ3-AGIP were prepared by ligation of the BamHII-digested PCR fragments containing both the start and the stop codons into the BamHII/Sall site of pFSZ3-Vpr (Fig. 2). This strategy resulted in duplication of the sequence from Sall to the end of the Vpr ORF in the vector. However, this duplication does not affect the amino acid sequence of the synthesized dR or rAG, because a termination codon is contained in the PCR DNA. dR PCR was performed with the template p842ZD861.1 (20) (GenBank™ accession number U88822) and primers P7/P8, and rAG PCR with the template p92NG083.2 (20) (GenBank™ accession number U88826) and primers P9/P10. dR/IP and rAG/IP mutant PCR were performed with a rabbit polyclonal antibody against His6-tagged Vpr following previously described conditions (19).

Performing analysis (ABI Prism) with primer P14, except for rD and rAG constructs, which were sequenced with P7 and P8, respectively.

**PCR Primer Pairs for Other Vpr Mutant Constructs Prepared by Ligations of the PCR DNA Fragments into the BamHII/Sall Sites of pFSZ3**—These primer pairs were as follows: I61A, P9/P15; L64A, P9/P16; L64V, P9/P17; L64M, P9/P18; L64P, P9/P19; L64Q, P9/P20; L64R, P9/P21; R62A/L64P, P9/P22; R63A/L64P, P9/P23. The DNA was eluted in 50 μl of RNase A for 30 min, and electrophoresed on a 1.5% mini agarose gel (with 4 μg/ml of RNase A). Western blot of cell extracts was performed with the Blood Mini Kit (Qiagen) and the region from the Rev-responsive element (RRE) to the beginning of Nef was preserved.

**Nef-LTR regions** are replaced with the polyadenylation signal (PA) from the SV40 T antigen. The 5′-untranslated region of Gag is maintained to preserve the splicing site for expression of Tat, Rev, and Gag. The original HIV-1 clone used is p99.6 (23). B, list of Vpr mutants with single- and double-point mutations expressed in the pFSZ3 vector. **Underlined sequences** represent the three helical regions identified by NMR studies (22). C, effects of Leu^64^ mutations on pro-apoptotic activity of Vpr. Vpr^63^ cells were transfected for 2 days in 12-well plates. Total cellular DNA, which included DNA from both apoptotic and normal cells, was prepared and examined by electrophoresis on a 1.5% agarose gel followed by staining with Vistra Green (Amersham Biosciences) and fluorescence microscopy analysis on a Storm 840 (Amersham Biosciences). M, 100-bp ladder marker (Promega). Lower panel, Western blot of cell lysates with a rabbit anti-Vpr antibody. D, Vpr amino acid residues 55–81 arranged by the helical wheel model. Asterisks indicate the residues mutated in the context of VprL64P (panel E). Arrow indicates position of Leu^64^ residues. **Position of Leu^64^**. Effects of secondary mutations on the pro-apoptotic potential of VprL64P. Small molecular weight DNA, which was formed only in apoptotic cells, was molecular weight DNA, which was formed only in apoptotic cells, was

**Based on the recent NMR structural information on Vpr in organic solvent, Vpr has flexible N-terminal and C-terminal domains and three helical domains arranged in a nonplanar U-shape (see Fig. 1B; helical domains are underlined) (22).** To examine the biological activities of Vpr, the wild type Vpr and its mutants with mutations in the different structural domains were expressed from the pFSZ3 vector (Fig. 1, A and B). This vector was based on the HIV-1 genome with targeted gene deletions and the insertion of the GFP coding sequence in place of the Env gene. All of the Vpr mutant constructs, as well as the wild type Vpr construct, were generated by PCR followed by sequence confirmation of the PCR-amplified region in the constructs. Vpr-expressing plasmids were individually transfected

**RESULTS**

Based on the recent NMR structural information on Vpr in organic solvent, Vpr has flexible N-terminal and C-terminal domains and three helical domains arranged in a nonplanar U-shape (see Fig. 1B; helical domains are underlined) (22). To examine the biological activities of Vpr, the wild type Vpr and its mutants with mutations in the different structural domains were expressed from the pFSZ3 vector (Fig. 1, A and B). This vector was based on the HIV-1 genome with targeted gene deletions and the insertion of the GFP coding sequence in place of the Env gene. All of the Vpr mutant constructs, as well as the wild type Vpr construct, were generated by PCR followed by sequence confirmation of the PCR-amplified region in the constructs. Vpr-expressing plasmids were individually transfected...
into human embryonic kidney cell line 293. Interestingly, after 18 h of transfection, extensive cell death, as indicated by cell rounding and detachment from the culturing surface, was observed for only the VprL64A mutant of the total of 22 Vpr proteins examined (data not shown). The wild type Vpr, as well as the Vpr-null construct (TAA), did not induce detectable cell death by this analysis.

Because degradation of chromatin DNA into fragments of 200-bp increments is the defining feature of apoptosis, total cellular DNA, which includes DNA from apoptotic and healthy cells, was prepared after 40 h of transfection and examined by electrophoresis on a 1.5% agarose gel (Fig. 1C). A strong apoptotic DNA ladder was observed only for the L64A mutant (Fig. 1C, lane 3) but not for the wild type Vpr (lane 2) or the other mutants listed in Fig. 1B (data not shown). Because apoptosis causes DNA fragmentation into various lengths, lane 3 (Fig. 1C) appears to have more total DNA than other lanes. However, quantification of the DNA suggests similar quantities of DNA in all the lanes. Because the pFSZ3 expression vector also expresses GFP and a truncated Vpu, the pFSZ3-VprL64A construct was subsequently modified to remove the GFP coding sequence and eliminate the translational start codon for Vpu. This modified VprL64A expression construct was as efficient as the original one in inducing apoptosis (data not shown).

HIV-1 is well known to generate mutations during its life cycle through the function of reverse transcriptase. However, the L64A mutation (codon: GCN) requires simultaneous mutations of the first two bases of the Leu64 codon CTG, rendering the L64A mutation difficult to occur in vivo. Leu64 is one of the most conserved site in HIV-1 Vpr (16). Among all of the single-base mis-sense mutations of the Leu64 codon CTT that are more likely to occur in vivo, L64P, L64Q, and L64V have been reported in GenBank™ (Table I). The rarity of these mutations is consistent with a critical role of Leu64 for Vpr function.

To analyze the effects of Leu64 mutations due to single-base changes on the apoptotic activity of Vpr, L64V, L64M, L64P, L64Q, and L64R mutants (Table I) were all expressed in the pFSZ3 vector and examined for their abilities to induce apoptosis (Fig. 1C). Two of these mutants, VprL64P (Fig. 1C, lane 6) and VprL64R (lane 8), had efficient pro-apoptotic activity, whereas other mutants, VprL64V, VprL64M, and VprL64Q, had no discernable differences from the wild type Vpr. Thus, single-base mutations of the Leu64 codon can enhance the pro-apoptotic potential of Vpr as efficiently as the L64A mutation.

In the Vpr NMR structure, Leu60 and Ile63 are shown to be part of a hydrophobic cluster formed between the end of helix II and the beginning of helix III (22). In the helical wheel model of Vpr helix III, Leu60 and Ile63 are on opposite sides of Leu64 (Fig. 1D). To examine the roles of the Leu64-proximal residues for the pro-apoptotic activity of VprL64P, secondary mutations L60A, I61A, R62A, and I63A were engineered into VprL64P. After transfection, small molecular weight DNA, which was formed only in apoptotic cells, was analyzed and quantified (Fig. 1, E and F). Among these secondary mutations, I61A reduced the pro-apoptotic ability of VprL64P by ~50% (Fig. 1E, lane 5), whereas the other three mutations had no significant

### Table I

| Codon | GenBank™ no. | Codon | GenBank™ no. |
|-------|--------------|-------|--------------|
| CTG   | (Wild type)  | CTG   | (Wild type)  |
| TGT   | None         | CCG   | (P)          |
| ATG   | None         | CCG   | (Q)          |
| TGG   | ND           | CCG   | (R)          |

| First base | Second base |
|------------|-------------|
| CTG        | CTG         |
| TGT (V)    | A275065     |
| ATG (M)    | None        |
| TGG (L)    | ND          |

Pro-apoptotic activities of Vpr molecules from different HIV-1 subtypes. A, alignment of three Vpr sequences: Vpr from a subtype B HIV-1 isolate (23); rD, Vpr from subtype D; rAG, Vpr from an A/G subtype recombinant (20). Asterisks indicate residues in rAG that are nonconservatively different from Vpr. B, expression of rD, rAG, their L64P mutants, and chimeric proteins between VprL64P and rAGP. The ORFs for rD, rAG, and their counterparts, rD/P and rAG/P, were PCR-amplified and cloned into pFSZ3-Vpr. The Tat start codon in the PCR was abolished (asterisk) without affecting Vpr amino acid residues. Solid arrow, position of the Tat ATG start codon in the vector. Right, L64P-N-AG and L64P-C-AG chimeric proteins containing the N- and C-terminal regions of rAG/P, respectively. C, L64P unmasks the pro-apoptotic potential of rD but not that of rAG. Small molecular weight DNA, which was formed only in apoptotic cells, was prepared and analyzed as described in Fig. 1E legend. In the Western blot (lower panel), rD, rAG, rD/P, and rAG/P migrated faster than Vpr for unknown reasons (compare lanes 2 and 3 with lanes 4–7). However, for rAG the determinant for a faster mobility seems to be localized in the C-terminal region (compare lanes 8 and 9). D, quantification of data in C. Conditions were the same as described in legend for Fig. 1E.
It has been shown that rAG is a hybrid Vpr molecule from an HIV-1 isolate of the A/G subtype recombinant (20). Although the exact breaking point for recombination is unclear, the N- and C-terminal regions of rAG are from the A and G subtypes, respectively. The nonconservative amino acid changes in rAG that are different from Vpr and rD reside mostly in helix II and the C-terminal domain (Fig. 2A). To examine the sequence determinants in rAG that render rAG/IP ineffective in apoptosis, the region of amino acids 1–63 was swapped between VprL64P and rAG/P (Fig. 2B, right) to obtain L64P/N-AG and L64P/C-AG chimeric proteins, which contain the N- and the C-terminal regions of rAG, respectively. Unexpectedly, when these chimeric proteins were expressed, both induced apoptosis effectively (Fig. 2C, lanes 8 and 9). These results suggest that the N- and C-terminal halves of rAG cooperate with each other to resist the apoptosis-enhancing effect of the L64P mutation.

**DISCUSSION**

This report describes the identification of Leu$^{64}$ as one of the key determinants for the potent pro-apoptotic potential of HIV-1 Vpr (Fig. 1). Substitution of Leu$^{64}$ with nonconservative residues Pro, Ala, and Arg enhances the pro-apoptotic potential of Vpr dramatically. It is possible that other nonconservative changes of Leu$^{64}$ also promote the pro-apoptotic potential of Vpr. This result is reminiscent of SIVmac239 Nef, which upon acquiring two point mutations promotes efficient viral replication in resting cells (24). Although mutations occur frequently during HIV-1 infection, Leu$^{64}$ mutations are rarely observed (Table I). This is consistent with a pivotal role of Leu$^{64}$ for the function of Vpr. It can be hypothesized that during the course of a natural HIV-1 infection, mutations in Vpr, including Leu$^{64}$ mutations, can emerge, resulting in Vpr molecules with different pro-apoptotic potentials. L64P-type mutations, however, may be selected against because of their potent pro-apoptotic activities.

The results in this study also suggest that Vpr molecules from different HIV-1 subtypes have different pro-apoptotic potentials (Fig. 2). Subtype-specific Vpr activity has not been reported. When examined for apoptosis induction, the Vpr from a subtype B HIV-1 has a moderate activity, whereas the Vpr from a subtype D HIV-1 has no detectable activity (Fig. 2). Interestingly, the L64P mutation enhances the pro-apoptotic potentials of both Vpr molecules (Fig. 2). In geographic areas where different subtypes of HIV-1 exist, genetic recombinations occur frequently (20, 25). These genetic recombinations may generate HIV-1 strains with different pathogenic properties (20, 25). In fact, the ancestor of HIV-1, SIVcpz, appears to be derived from genetic recombinations among SIV isolates that infect various species of monkeys (26). Interestingly, a hybrid Vpr molecule from the A/G subtype recombinant HIV-1 resists the apoptosis-promoting effect of the L64P mutation, although the original A/G hybrid Vpr has a moderate pro-apoptotic activity (Fig. 2). Thus, the pro-apoptotic activity of Vpr may be affected by subtype-specific sequences, point mutations during HIV-1 replication, or genetic recombination between different HIV-1 isolates. More thorough analysis in this regard may lead to a better understanding of the roles of Vpr during pathogenesis caused by different subtypes of HIV-1.

The recently determined NMR structure of Vpr has three helical regions (Fig. 1B). The third helix, where Leu$^{64}$ resides, is most abundant in Leu/Ile residues and is capable of forming a leucine zipper-like structure (27). The structure of Vpr is compact because of tertiary hydrophobic interactions between helix II and helix III and potential long-range interactions between helix I and helix III under physiological conditions (22). It has been noted that Leu$^{60}$ and Ile$^{63}$ are both part of a hydrophobic cluster formed between helix II and helix III (22). However, mutation of either residue does not affect the strong pro-apoptotic activity of VprL64P (Fig. 1E). Although both Leu$^{60}$ and Ile$^{63}$ are located in the vicinity of Leu$^{64}$ in the Vpr helical wheel model (Fig. 1D), only I61A inhibits the strong pro-apoptotic activity of VprL64P. It is possible that the effects of the L60A and I63A mutations are compensated for by the nearby Leu/Ile residues in helix III (Fig. 1D). Alternatively, pairing of Leu/Ile residues in the helical wheel model of helix III (Fig. 1D) may be critical for Vpr interaction with the cellular apoptosis regulatory pathways.

Apoptosis induction by VprL64P is dependent on caspase(s) (data not shown), similar to previous observations on the wild type Vpr (12, 17, 28). Caspase-dependent apoptosis is initiated by the activation of the initiator caspases, including caspases 8 and 9, which in turn activate the effector caspases (29). Although caspase 8 is essential for the death receptor-mediated apoptosis pathway (30), caspase 9 is activated by the Bcl-2 family-controlled mitochondrial pathway (29). In certain types of cells, apoptosis induction by activated caspase 8 requires amplification through the mitochondria-caspase 9 pathway (31). It has been reported that the adenoviral-expressed wild type Vpr activates caspase 9 through cytochrome c release from the mitochondria (17). Cell-expressed Vpr has also been shown to cause activation of caspase 8 and mitochondrial permeabilization (18). Thus, it remains unclear which initiator caspase is essential for Vpr induction of apoptosis. Identification of the initial biochemical events responsible for caspase activation by Vpr and VprL64P may help us to better understand the mechanism of Vpr-induced apoptosis.

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