Multifactorial role of HIV-Vpr in cell apoptosis revealed by a naturally truncated 54aa variant

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Although antiretroviral therapy (ART) is effective at suppressing the human immunodeficiency virus type 1 (HIV-1) replication, HIV-1 infection is still a global public health problem. HIV-1 accessory protein viral protein R (Vpr) is a multifunctional protein with a primary role in regulating cellular apoptosis.[1] The amino acid (aa) positions 52–96 in the C-terminus of Vpr were identified as the apoptosis-regulating domain.[2,3] In this study, we found a natural Vpr variant truncated at the 54 aa position (*54Vpr) from HIV patients in the acquired immune deficiency syndrome (AIDS) phase that mediated both pro- and antiapoptotic cellular effects by interacting with distinct adenine nucleotide translocator (ANT) isoforms. A novel apoptosis-regulating domain was further identified in the 23–37 aa position in the N-terminus of Vpr.

The truncated CRF07_BC *54Vpr and intact Vpr (XJN0084_54W) were from the previously described XJN0084 isolate,[4] while the B subtype *54Vpr (pNL4-3_54Stop and XJN0084_54W) or *54Vpr (pNL4-3_54Stop and XJN0084) did not show any significant changes in Annexin-V cells (Figure 1B and C, P > 0.05). In contrast, both wildtype (pNL4-3, XJN0084_54W) and *54Vpr (pNL4-3_54Stop and XJN0084) had significantly pro-apoptotic effects in TZM-bl cells compared to the GFP vector control (Figure 1B and C, P < 0.05). Moreover, these results demonstrated that *54Vpr (pNL4-3_54Stop and XJN0084) showed similar proapoptotic activity to wildtype (pNL4-3, XJN0084_54W) (P > 0.05) and also suggested that the effects of Vpr to induce apoptosis appear to be cell line dependent.

It has been proposed that Vpr binds to ANT expressed in the mitochondrial membrane to induce a caspase-dependent apoptotic pathway.[5] To explore the underlying mechanism of the cell-dependent proapoptotic activity of Vpr, we analyzed the RNA expression levels of three different ANT isoforms (ANT1–3) in 293T and TZM-bl cells using X-treme GENE HP (Roche, Basel, Switzerland). After Annexin-V staining using the Annexin V-APC Apoptosis Detection Kit (eBioscience, San Diego, CA, USA), fluorescence-activated cell sorting (FACS) analysis was performed in triplicate to detect apoptosis 293T and TZM-bl cells transfected with Vpr and/or ANT expression vectors. Expression of Vpr and ANT proteins in transfected cell lines was subsequently confirmed by Western blot analysis (data not shown). Statistical differences were determined by paired Student’s t-test for paired comparisons and one-way ANOVA with posttests for multiple group comparisons using Prism 5.02 (GraphPad Prism, Version 5.02, La Jolla, CA, USA).

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cell lines using an SYBGreen kit (Promega, Madison, WI, USA) (the primers were as follows: ANTI1: 5'-TGCTGATGTTGGGCAAGGCGC-3' and 5'-GCCCTTGCTCTTCAGGAAGG-3'; ANTI2: 5'-GGTAAAGCTGGAGCTGAAAGG-3' and 5'-TTGGCTCCTTCATCACG-3'; ANTI3: 5'-GGGAAAGTCAGGCACAGAGCGTACAGGACCAGCACGAGG-3'). The quantitative PCR analysis showed that the expression of ANTI3 in TZM-bl cells was markedly higher than the levels in 293T and HeLa cells [Figure 1D, P < 0.05], and the expression levels of ANTI1 and ANTI2 were extremely low in all three cell lines (accounting for less than 1% of ANTI3).

Considering the pro-apoptotic role of ANTI3, although further studies are required, the data may partially explain why Vpr could not induce cell apoptosis in 293T cells but did in TZM-bl cells, suggesting that the pro-apoptosis activity of Vpr relied on the expression level of ANTI isoforms in cells.

To further clarify the role of the ANTI protein during the proapoptotic effects of Vpr, ANTI1-3 expression vectors and wildtype GFP-Vpr were cotransfected into 293T or TZM-bl cells, respectively. As shown in Figure 1E and 1F, GFP-Vpr transfection resulted in a significant increase in the apoptosis percentage of GFP-positive 293T cells in the presence of ANTI1 or ANTI3 (P < 0.05). In contrast, GFP-Vpr resulted in a significantly decreased apoptosis percentage in GFP-positive TZM-bl cells in the presence of ANTI2 (Figure 1G, P < 0.05). Moreover, the interaction between ANTIs and Vpr was further identified in the
co-immunoprecipitation (Co-IP) analysis [Figure 1H]. These data indicated that the proapoptotic activity of Vpr is dependent on distinct ANT protein expression in various cell lines.

The identified proapoptotic effects of the truncated 54Vpr as described earlier suggest the presence of a potential new cell death domain in the N-terminus of Vpr. To characterize the new proapoptotic motif, a series of site-directed mutations of the GFP-Vpr were introduced, as described in Figure 1I. These nonsense-mutated GFP-Vpr expression vectors at aa positions 43, 38, 34, 30, 27, and 23 were subsequently transfected into TZM-bl cells and evaluated for their proapoptotic activities. Cells transfected with the mutated Vpr truncated at aa positions 34, 38, and 43 all showed significantly higher apoptosis percentages in GFP-positive cells compared with those transfected with the GFP vector control (Figure 1J, *P < 0.05*). In contrast, cells transfected with Vpr mutants truncated at aa positions 30, 27, and 23 showed no significant differences in apoptosis percentage in GFP-positive cells compared with the GFP vector control (Figure 1J, < 10% vs. < 5%, *P > 0.05*). Meanwhile, truncated Vpr containing the intact first helix (pNL4-3.38stop and pNL4-3.43stop) exhibited comparable proapoptotic activity to *54Vpr* and wildtype Vpr (*P > 0.05*). In summary, these results suggested the presence of a potential cell death domain located in the intact first helix (position 23aa to 37aa) in the N-terminus of Vpr.

Based on the aforementioned results, we further explored whether the Vpr23-37aa domain in the N-terminus is a death domain responsible for the proapoptotic activity. Analysis of the percentage of Annexin V+PI+ in GFP-positive cells is presented in Figure 1K. The Vpr23-37 aa and the well-known cell death domain (71–92 aa) in the C-terminus of Vpr had significantly higher proapoptotic activity than the pNL4-3.23StopVpr and GFP vector control. Interestingly, one previous study predicted a first helix (pNL4-3.38stop and pNL4-3.43stop) to *54Vpr* and wildtype Vpr (*P > 0.05*). In summary, these results suggested the presence of a potential cell death domain located in the intact first helix (position 23aa to 37aa) in the N-terminus of Vpr, which was confirmed by the present data.

Several studies have confirmed that Vpr plays multiple roles via several critical motifs: the N-terminal 42 aa of Vpr include a helix 1 and constitute the oligomerization domain of the protein[3,7–9] and have been associated with the cytopathic functions of Vpr; the C-terminal moiety (Vpr 52–96 aa) containing helix 3 binds to ANT and induces apoptosis via a highly conserved leucine-rich domain (Vpr 71–92 aa), which is widely recognized as the cell death motif.[10] The study presented here demonstrated that the *54Vpr* variant has the same activity as wildtype Vpr in regulating apoptosis in cell lines and further identified a novel proapoptotic motif (23–37 aa) located in the first helix of the Vpr protein. Furthermore, the study also revealed the multifactorial roles of HIV-1 Vpr in regulating cell apoptosis via interactions with different ANT isoforms. The data presented here indicated that the overexpression of ANT1 or ANT3 promoted Vpr-mediated cellular apoptosis, whereas the overexpression of ANT2 inhibited cellular apoptosis. Therefore, we not only provided novel evidence that the ANT proteins play a critical role in the Vpr-mediated apoptotic mechanism but also proposed a new theory that proapoptotic or antiapoptotic effects of Vpr are dependent on the distinct expression patterns of ANT isoforms in cells. As a multifactorial protein, Vpr plays critical roles during the HIV-1 life cycle; however, the contributions of interactions between ANT isoforms and Vpr to HIV-1 persistent or latent infection still need further elucidation.

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**Conflicts of interest**

None.

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