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 the wildtype Vpr were from pNL4-3 [Figure 1A]. The full-length gene-encoding region for *ANT1–3* was amplified using the following primers: *ANT1*-F: 5'-CCCGAATTCTCACCATGGGTGATCAC GCTTGG-3', *ANT*1-R: 5'-AGAAAGCTTGACATATTT TTTGATCTCAT-3'; *ANT2*-F: 5'-CCCGAATTCTCAC-CATGACAGATGCCGCTGTG-3', *ANT2*-R: 5'-AGA AAGCTTTGTGTACTTCTTGATTTCAT; *ANT3*-F: 5'-CCCGAATTCTCACC ATGACGGAACAGGCCATC-3', *ANT3*-R: 5'-AGAAAGCTTGATCACCTTCTTGAGCTC GT-3'. Green fluorescent protein (GFP)-Vpr expression vectors were constructed using the pEGFP-C3 vector, and *ANT1-3* expression vectors were constructed using the pCMVTNT-3xFlag vector, which was used to transfect into 293T cells 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).

The 293T cells transfected with wildtype Vpr (pNL4-3, 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 proapoptotic effects in TZM-bl cells compared to the GFP vector control (Figure 1B and 1C, $P < 0.05$). 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

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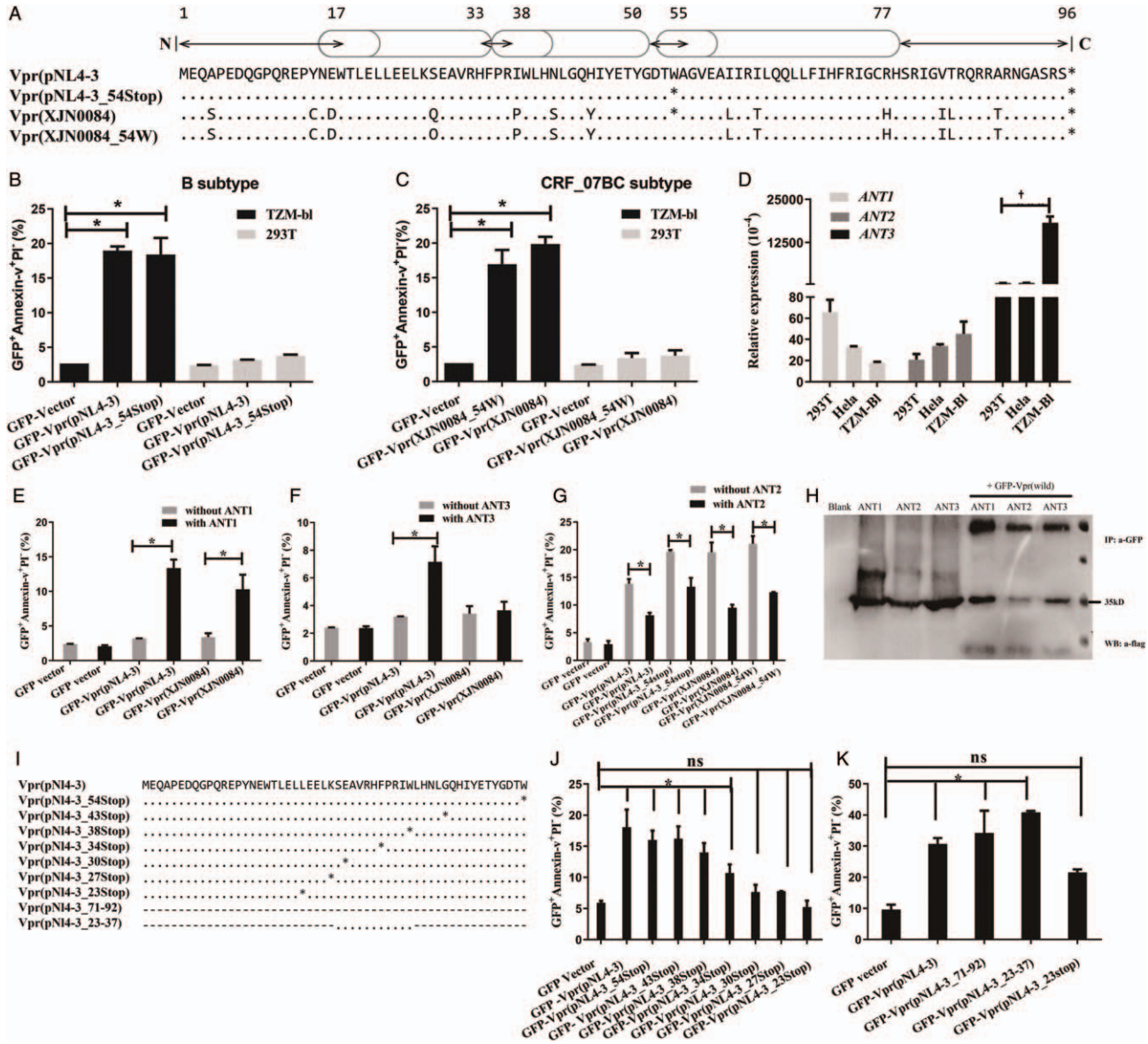

Figure 1: Analysis of apoptosis effects induced by Vpr. (A and I) Amino acid sequences of wildtype Vpr, *54Vpr, and site-mutated Vpr. (B, C, E–G, J, and K) Apoptosis assay by FACS. The percentage of Annexin-V + PI- in GFP-positive cells was determined by flow cytometry. Results are representative of data for samples from duplicate measurements. The results are representative of data for samples, and significant differences from the control were calculated by Student's *t* test (*means $P < 0.05$; †means $P < 0.01$). (D) Quantitative PCR analysis of the RNA expression of *ANT* isoforms in cell lines. The values were normalized using GAPDH as a reference. (H) Co-IP analysis of cotransfected GFP-Vpr and *ANT1*, *ANT2*, and *ANT3*. The capture antibodies were anti-GFP antibodies, and the detection antibodies were anti-flag antibodies. ANT: Adenine nucleotide translocator; FACS: Fluorescence-activated cell sorting; Co-IP: Co-immunoprecipitation; GFP: Green fluorescent protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ns: Not significant; Vpr: Viral protein R.

cell lines using an SYBGreen kit (Promega, Madison, WI, USA) (the primers were as follows: *ANT1*: 5'-TGCT GATGTGGGCAAGGGCGC-3' and 5'-GCCTTGGCTC CTTCGTCTTTT-3'; *ANT2*: 5'-GGTAAAGCTGGAGC TGAAAGG-3' and 5'-TTG CCTCCTTCATCACGA-3'; *ANT3*: 5'-GGGAAAGTCAGGCACAGAG CG-3' and 5'-CGTACAGGACCAGCACGAAGG-3'). The quantitative PCR analysis showed that the expression of *ANT3* 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 *ANT1* and *ANT2* were extremely low in all three cell lines (accounting for less than 1% of *ANT3*). Considering the proapoptotic role of *ANT3*,[6] 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 *ANT* isoforms in cells.

To further clarify the role of the *ANT* protein during the proapoptotic effects of Vpr, *ANT1-3* 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 *ANT1* or *ANT3* ($P < 0.05$). In contrast, GFP-Vpr resulted in a significantly decreased apoptosis percentage in GFP-positive TZM-bl cells in the presence of *ANT2* (Figure 1G, $P < 0.05$). Moreover, the interaction between *ANT*s 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 controls. Interestingly, one previous study predicted a potential cell death domain in the N-terminus of Vpr,[5] which was confirmed by the present data.

Several studies have confirmed that Vpr plays multiple roles via several critical motifs: the N-terminal 42 aas 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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