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Human Immunodeficiency Virus Type 1 Vpr Modulates Cellular Expression of UNG2 via a Negative Transcriptional Effect▽

Christelle Langevin,[1,2] Priscilla Maidou-Peindara,[1,2] Per Arne Aas,[3] Guillaume Jacquot,[1,2] Marit Otterlei,[3] Geir Slupphaug,[3] and Serge Benichou[1,2]*

Institut Cochin, CNRS UMR 8104, Université Paris Descartes, Paris, France[1]; INSERM U567, Paris, France[2]; and Department of Cancer Research and Molecular Medicine, Norvegian University of Science and Technology, Trondheim, Norway[3]

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It was recently reported that human immunodeficiency virus type 1 (HIV-1) Vpr induced the proteasomal degradation of the nuclear UNG2 enzyme for efficient virus replication. We confirm here that HIV-1 infection and Vpr expression reduce the level of endogenous UNG2, but this effect is not reverted by treatment with the proteasome inhibitor MG132. Moreover, this reduction is not mediated by Vpr binding to UNG2 and is independent of the Vpr-induced G_2 arrest. Finally, we show that Vpr influences the *UNG2* promoter without affecting *UNG1* gene expression. These data indicate that the Vpr-induced decrease of UNG2 level is mainly related to a transcriptional effect.

Among the auxiliary proteins of human immunodeficiency virus type 1 (HIV-1), Vpr is a highly conserved regulatory protein of 96 residues (14 kDa). The structure of Vpr has been determined and consists of a hydrophobic central core domain with three well-defined α-helices surrounded by flexible N- and C-terminal domains (16). In contrast to other HIV-1 auxiliary proteins, Vpr is specifically incorporated into virions (17, 24), in accordance with its requirement in the early phases of viral replication (29, 30). Vpr contributes both to reverse transcription of the viral RNA (3, 15) and to nuclear transport of the proviral DNA (2, 5). In addition, Vpr displays other activities, including an arrest of the cell cycle at the G_2/M transition, the induction of apoptosis, and transcriptional effects on the HIV-1 long terminal repeat, as well as host cell genes (1, 13).

These Vpr functions have been linked to interactions with cellular partners, including the nuclear form of uracil DNA glycosylase (UNG2), an enzyme that removes uracil formed either by misincorporation of dUMP during replication or by deamination of cytosine (12). UNG is a key component of DNA repair mechanisms either in the nucleus or in the mitochondria through involvement of specific isoforms (UNG2 and UNG1, respectively) (18). Although the determinants involved in the interaction between Vpr and UNG2 have been delineated (23), controversial models regarding the role of UNG2 during HIV-1 replication have been proposed (11, 15, 20, 22). Although several reports initially indicated that Vpr-mediated incorporation of UNG2 into HIV-1 virions was required to modulate the virus mutation rate and for efficient virus replication (3, 15), recent reports suggest that UNG2 encapsidation has a detrimental effect on virus replication (22). The later model proposes that Vpr induces the proteasomal degradation of UNG2 in virus-producing cells. Since these studies were performed with overexpressed UNG2, we reexamined the influence of HIV-1 infection and Vpr expression on the level of endogenous UNG2.

HIV-1 infection reduces the level of endogenous UNG2 protein. The level of the endogenous UNG2 protein was first analyzed in HeLa-CD4 cells infected with the HIV-1NL43 strain. Cell lysates were prepared at the indicated times postinfection and proteins were analyzed by Western blotting (WB) using anti-UNG polyclonal antibody (ab23926; Abcam) directed against both UNG1 and UNG2 isoforms or with anti-p24 (provided from the NIH AIDS Research and Reference Reagent Program) directed against the viral capsid. Before infection, UNG proteins were easily detected in cell extracts, and we could distinguish two bands of 37 and 31 kDa corresponding to UNG2 and UNG1, respectively (Fig. 1A, mock), whereas a progressive decrease in the amount of UNG2 was observed from 24 h postinfection (Fig. 1A and B, left panel). In contrast, the amount of UNG1 was not significantly different from that detected in uninfected cells, even by 3 days postinfection. In the same time, the amount of p24 protein increased as a result of de novo virion synthesis (Fig. 1A). The fact that we observed a sharp decrease in the level of UNG2 protein after infection, whereas the actin and UNG1 levels remained unchanged (Fig. 1A), argues for a specific effect of HIV-1 in decreasing the UNG2 level in infected cells.

Next, we explored whether the decrease of UNG2 level observed during HIV-1 infection was related to Vpr expression. Cells were infected with either wild-type or ΔVpr HIV-1NL4-3, and cell lysates were analyzed (Fig. 1A). As demonstrated in Fig. 1B, the UNG2 signal disappeared upon infection with wild-type HIV-1, whereas a slight decrease of UNG2 was observed in HIV-1ΔVpr-infected cells. The same Vpr-dependent results were obtained by immunofluorescence analysis of UNG2 performed on either HeLa-CD4 or HPB-ALL T cells infected with wild-type or ΔVpr HIV-1, and using anti-UNG PU59 antibody (6) (Fig. 2). Together, these results demonstrate that the level of the endogenous nuclear UNG2 protein is markedly reduced in a Vpr-dependent manner in HIV-1-infected cells.

Vpr is sufficient to induce reduction of UNG2 protein. To further document the role of Vpr in these observations, the

* Corresponding author. Mailing address: Institut Cochin, 27 Rue du Faubourg Saint-Jacques, 75014 Paris, France. Phone: (33) 1 40 51 65 78. Fax: (33) 1 40 51 65 70. E-mail: serge.benichou@inserm.fr.

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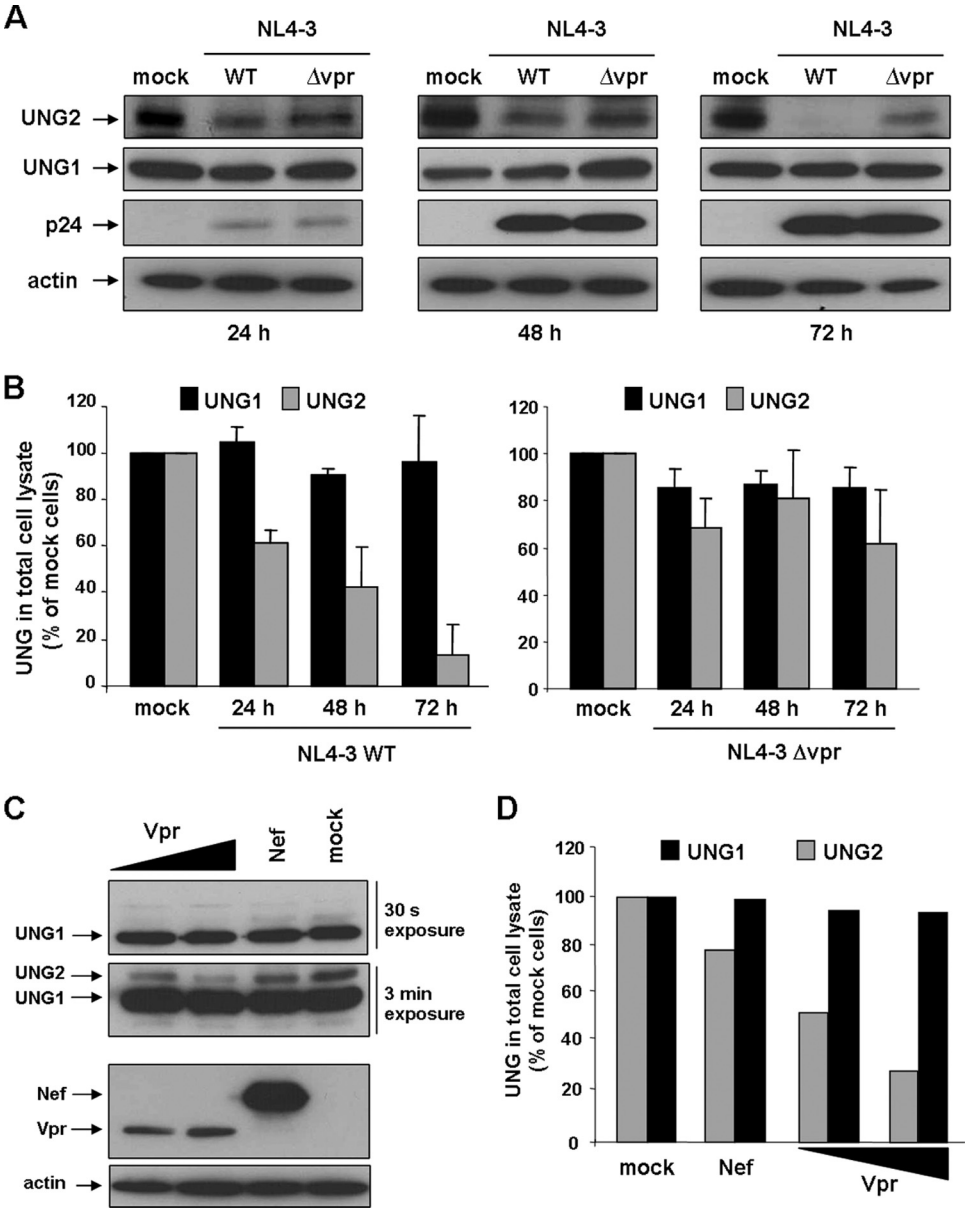

FIG. 1. Downregulation of the UNG2 protein in HIV-1-infected cells and in Vpr-expressing cells. (A) Immunoblot analysis of UNG proteins in HIV-1-infected cells. HeLa-CD4 cells were infected with VSVG-pseudotyped wild type or ΔVpr HIV-1NL43, and protein extracts were analyzed before (mock) or after infection (24, 48, and 72 h) by WB using anti-UNG1 and -UNG2 polyclonal antibody PU59 (upper panels), anti-p24 (middle panels), and anti-actin antibodies (lower panels). (B) The intensity of UNG2 and UNG1 bands was quantified by densitometry using NIH Images software from the panels shown in panel A. UNG1 and UNG2 levels were normalized to those of actin. The values represent the percentage of the UNG2 (▨) and UNG1 (■) signal intensity in wild type (left part)- or Δ*vpr* (right part)-infected cells relative to mock cells (100%) and correspond to the mean of three independent experiments. (C) Immunoblot analysis of UNG proteins in Vpr-expressing cells. HeLa cells were transfected with vectors for expression of HA-tagged forms of Vpr (0.5 or 1 μg of plasmid) or Nef (0.25 μg); mock, control cells transfected with the empty plasmid (1 μg). At 24 h after transfection, protein extracts (50 μg) were analyzed by WB using anti-UNG (PU59, upper panels), anti-HA (middle panel), and anti-actin (lower panel) antibodies. The immunoblot for analysis of UNG1 and UNG2 expression was exposed for 30 s (upper panel) or 3 min (lower panel). (D) The intensity of UNG2 and UNG1 bands was quantified by densitometry using NIH Images software from the panels shown in panel C. UNG1 and UNG2 levels were normalized to those of actin. The values represent the percentage of the UNG2 (▨) and UNG1 (■) signal intensity in Nef- and Vpr-expressing cells relative to mock-transfected cells (100%) and are representative of three independent experiments. WT, wild type.

level of UNG2 was analyzed in HeLa cells expressing increasing amount of Vpr fused to the hemagglutinin (HA) epitope (10). As controls, we used HA-tagged Nef expressed in the same plasmid or empty vector (mock). The levels of Vpr expression in transfected cells was similar to what we detected from the WB analysis of HeLa-CD4 infected cells using anti-Vpr antibody to detect both HA-Vpr and native Vpr in transfected and infected cells, respectively (data not shown). In cells

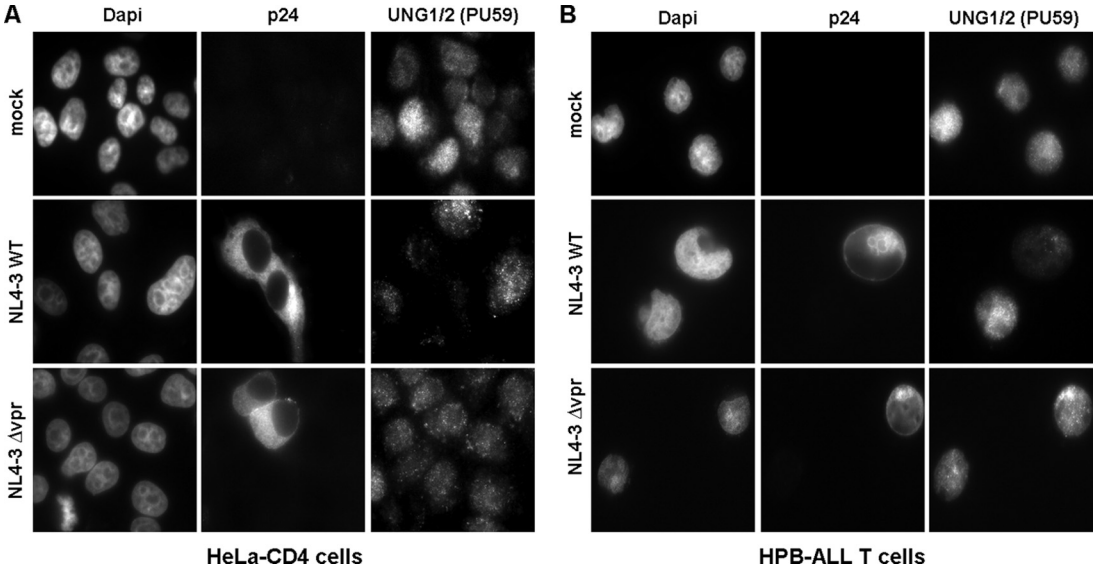

FIG. 2. Immunofluorescence analysis of UNG2 downregulation in HIV-1-infected cells. HeLa-CD4 (A) or HPB-ALL T (B) cells were infected with wild type (middle panels) or Δ*vpr* (lower panels) HIV-1NL43 and were then analyzed 48 h after infection by immunofluorescence. Cells were fixed, permeabilized, and subsequently stained with anti-UNG (PU59), anti-p24 antibodies and DAPI (4′,6′-diamidino-2-phenylindole). Cells were analyzed by epifluorescence microscopy, and images were acquired by using a charge-coupled device camera. WT, wild type.

expressing HA-Vpr, a reduced level of UNG2 was observed compared to mock-transfected or Nef-HA-expressing cells (Fig. 1C and D). This effect was specific to UNG2, since Vpr did not affect the level of UNG1. These results demonstrate that Vpr expression is sufficient to reduce the level of endogenous UNG2 in the absence of other viral proteins.

Because the transfection efficiency obtained for biochemical analysis did not exceed 50%, we also performed immunofluorescence analysis to focus on Vpr-expressing cells. HeLa cells were transfected with HA-Vpr or HA-IN expression vectors, fixed and permeabilized 24 h later before staining with anti-UNG (PU59) and anti-HA antibodies. As opposed to UNG1, which is constitutively expressed, the level of UNG2 is differentially regulated during the cell cycle (8, 25). Thus, the level of UNG2 is highest in late G_1/S, whereas it is degraded in late S/G_2 (6). Accordingly, the cytoplasmic mitochondrial UNG1 staining was constant in all cells, whereas the nuclear UNG2 signal had different intensity levels (Fig. 2A). In agreement with the biochemical approach, nuclear UNG2 staining with PU59 anti-UNG antibody disappeared in most of the Vpr-expressing cells; the same result was obtained using two other anti-UNG polyclonal antibodies directed against both UNG1 and UNG2 (PU1A) or specific of UNG2 (PU1sub) (data not shown). The number of UNG2-defective cells among the Vpr-transfected cells was quantified (Fig. 3B). In basal conditions (mock), ca. 40 to 50% of the cells were defective for UNG2, and this proportion remained unchanged in cells expressing HA-IN used as a negative control (28) (Fig. 3B and data not shown). In contrast, a large majority (95%) of Vpr-expressing cells were devoid of nuclear UNG2 expression. In Vpr-expressing cells, the UNG2/UNG1 staining ratio did not exceed 0.1, whereas this ratio remained between 0.4 and 0.5 in mock or HA-IN-expressing cells (Fig. 3B, right panel). Together, these results show that Vpr expression is sufficient for reduction of the endogenous UNG2.

Reduction of UNG2 is not related to Vpr binding or Vpr manipulation of the cell cycle. Since extensive studies have lead to a number of different biological functions associated with Vpr (1, 13), we investigated further the involvement of Vpr by analyzing the relationship between some of its biological properties and the reduction of the UNG2 level. Therefore, we used distinct Vpr mutants targeting different functions of HIV-1 Vpr.

First, we focused on the requirement of direct interaction between Vpr and UNG2. Since the tryptophan residue in position 54, located between the second and third helix of Vpr, is critical for interaction with UNG2 (23), the VprW54R mutant was used to challenge whether Vpr binding to UNG2 was required for reduction of UNG2 level. HeLa cells expressing either wild-type or W54R HA-Vpr proteins were analyzed by immunofluorescence. As illustrated in Fig. 3A, most of the cells expressing wild-type or W54R Vpr proteins displayed a strong decrease in nuclear UNG2 signal, and quantification showed that more than 95% of cells expressing the W54R mutant were defective for UNG2 expression (Fig. 3C). Surprisingly, this result indicates that the reduction of UNG2 protein is not related to Vpr binding to UNG2.

Since the turnover of UNG2 is finely regulated during the cell cycle (6, 8, 25), we then focused on a potential indirect regulation of UNG2 expression in Vpr-expressing cells, which could rely on the cytostatic property of Vpr. To test this hypothesis, two Vpr mutants (R90K and Q65R) deficient for G_2 arrest were analyzed (4, 10, 14, 23, 26). While mutations in the C-terminal region of Vpr, such as R90K, is known to abrogate Vpr-mediated G_2-arrest (10, 23), it was recently shown that the Q65R substitution led to a mutant that failed to bind the DCAF1 subunit of the Cul4a/DDB1 E3 ubiquitin ligase and resulted in an inactive protein (4, 14, 26). As illustrated in Fig. 3A and C, VprR90K and VprQ65R both affected intracellular levels of UNG2 to the same extent as the wild-type protein. Since it was reported that fusion of green fluorescent protein

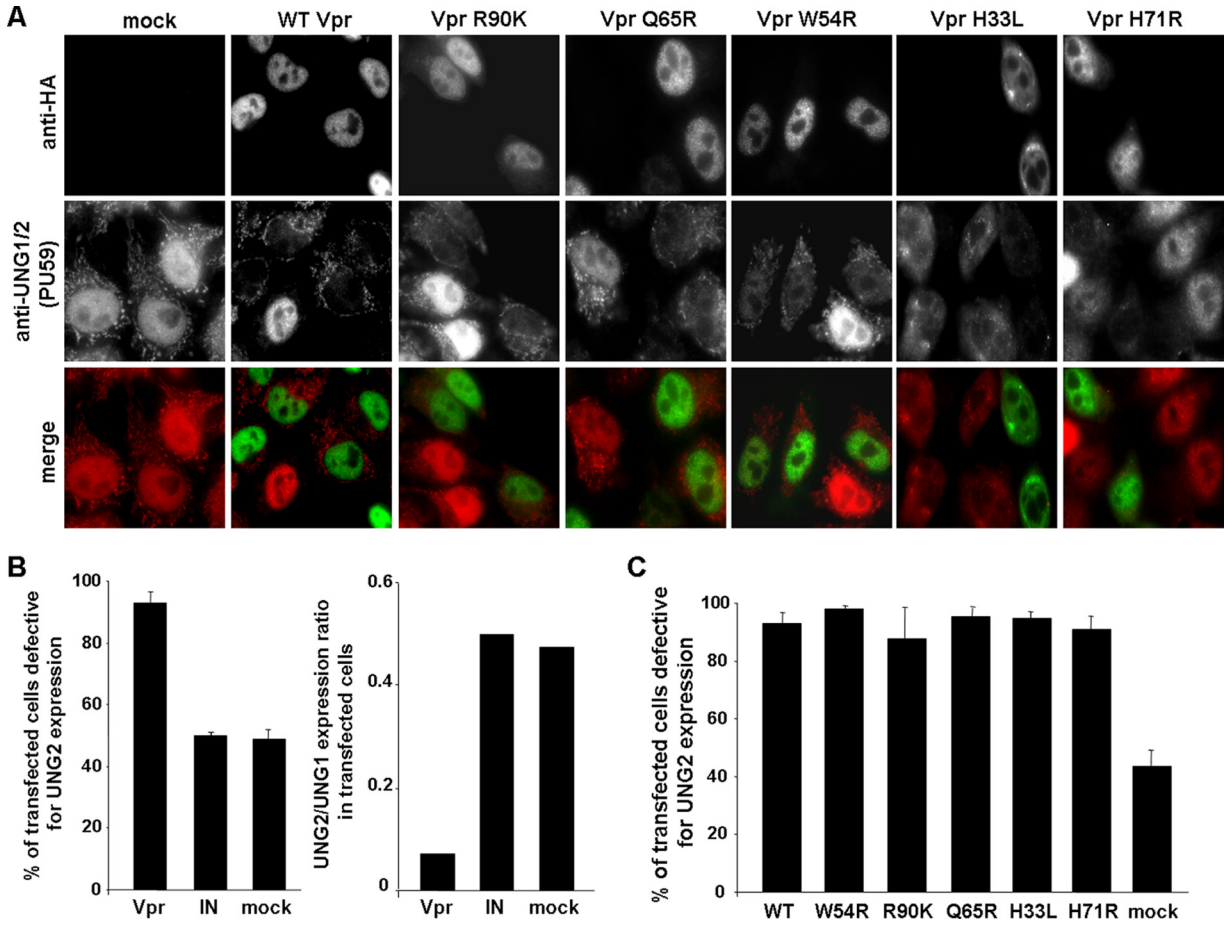

FIG. 3. Downregulation of the UNG2 protein is independent of Vpr binding and Vpr-induced G_2 arrest. (A) Immunofluorescence analysis of UNG1/2 expression in Vpr-expressing cells. At 24 h after transfection with vectors encoding wild-type, mutated HA-Vpr proteins or with the empty plasmid (mock), cells were analyzed by immunofluorescence as indicated in Fig. 2 with anti-HA (upper panels) and anti-UNG (PU59) (middle panels) antibodies. (B) Quantification of cell number defective for UNG2 expression among the transfected cell population. The number of cells with a reduced nuclear UNG2 staining was quantified over 100 cells expressing HA-Vpr, HA-IN, or the empty plasmid (mock). On the left side, the results are expressed as the percentage of cells showing defective nuclear UNG2 staining among the transfected cell population. Values are the means of three independent experiments. On the right side, ratio of UNG2/UNG1 expression quantified from transfected cells. Number of transfected cells expressing a nuclear UNG2 staining was divided by number of cells expressing a cytoplasmic UNG1 signal. Values are the means of three independent experiments. (C) The number of cells with a reduced nuclear UNG2 staining was quantified over 100 cells expressing wild-type or mutated HA-Vpr proteins. The results are expressed as indicated in panel B. Values are the means of three independent experiments. WT, wild type.

(GFP) to the N- or C-terminal end of Vpr result in fusion proteins that failed to induced G_2 arrest (1), we also examined expression of UNG2 in cells expressing GFP-Vpr or Vpr-GFP. We found that UNG2 staining decreased in the nuclei of both GFP-Vpr- and Vpr-GFP-expressing cells (data not shown). Finally, two additional Vpr mutants (H33L and H71R), which fail to bind UNG2 and are deficient for G_2 arrest (23), were also analyzed (Fig. 3A and C). Again, both mutants similarly affected nuclear UNG2 staining to the same extent as the wild-type protein. Together, these results strongly indicate that the reduction of UNG2 observed in Vpr-expressing cells is independent of the manipulation of the cell cycle by Vpr. In contrast to results reported by others (21), they also indicate that interaction of Vpr with the Cul4A/DDB1 E3 ligase is not required for reduction of endogenous UNG2.

Vpr-induced reduction of endogenous UNG2 is not related to proteasomal degradation. Because it was suggested that Vpr binding to UNG2 was able to trigger the proteasomal degradation of UNG2 (22), we further evaluated the influence of proteasome inhibitor treatment on the level of UNG2 in Vpr-expressing cells. At 24 h after transfection, cells were treated for 6 h with a 20 μM concentration of MG132 proteasome inhibitor (Fig. 4A). A similar decrease of UNG2 staining was observed by immunofluorescence in Vpr-expressing cells with or without MG132 treatment. Quantification indicated that 90 and 96% of the Vpr-expressing cells were defective for UNG2 in MG132-treated and untreated cells, respectively (Fig. 4C). Similarly, MG132 treatment did not restore the reduced UNG2 signal detected by WB in Vpr-expressing cells (Fig. 4B and D). As an internal control of MG132 treatment (7), endogenous β-catenin was stabilized in MG132-treated cells. Interestingly, we detected that MG132 treatment was associated with a higher HA-Vpr signal; this confirms that the level of Vpr is modulated by the cellular degradation machinery (14). Together, these results indicate that the mechanisms that regulate the endogenous UNG2 level in Vpr-expressing cells do not depend on activation of the proteasome.

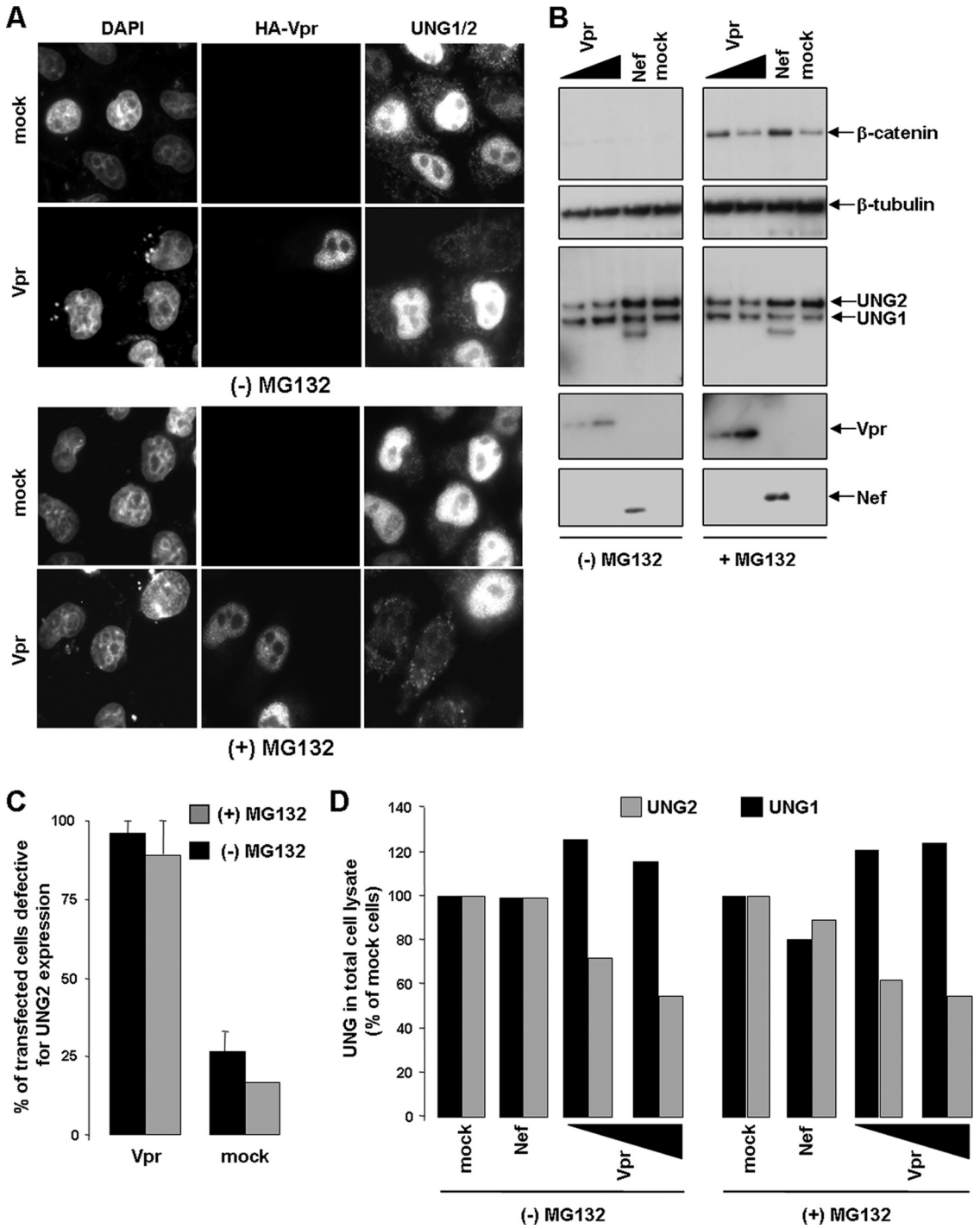

FIG. 4. Downregulation of the UNG2 protein is independent of the proteasome degradation. (A) Immunofluorescence analysis of UNG2 expression in Vpr-expressing cells treated with the MG132 proteasome inhibitor. At 24 h after transfection with vectors for expression of wild-type HA-Vpr or with the empty plasmid (mock), the cells were treated (lower panels) or not (upper panels) for 6 h with 20 μM MG132 and then analyzed by immunofluorescence as described in Fig. 3. (B) Immunoblot analysis of UNG2 expression in Vpr-expressing cells treated with MG132. Cells were transfected as described in Fig. 1C with vectors encoding HA-tagged forms of Vpr (0.5 or 1 μg of plasmid DNA) or Nef (0.25 μg); mock, control cells transfected with the empty plasmid (1 μg). Protein extracts were then analyzed by WB with anti-UNG (PU59), anti-HA for expression of the HA-tagged Vpr and Nef proteins, anti-β-catenin as a control of proteasome inhibition, and anti-β-tubulin antibodies. (C) The number of cells with a reduced nuclear UNG2 staining was quantified over 100 cells expressing HA-Vpr and treated (▨) or not (■) with MG132. The results are expressed as indicated in Fig. 3B. Values are the means of three independent experiments. (D) The intensity of UNG2 and UNG1 bands was quantified by densitometry using NIH Images software from the panels shown in panel B. UNG1 and UNG2 levels were normalized to those of β-tubulin. The values represent the percentage of the UNG2 (▨) and UNG1 (■) signal intensity in Nef- and Vpr-expressing cells relative to mock-transfected cells (100%), and are representative of three independent experiments.

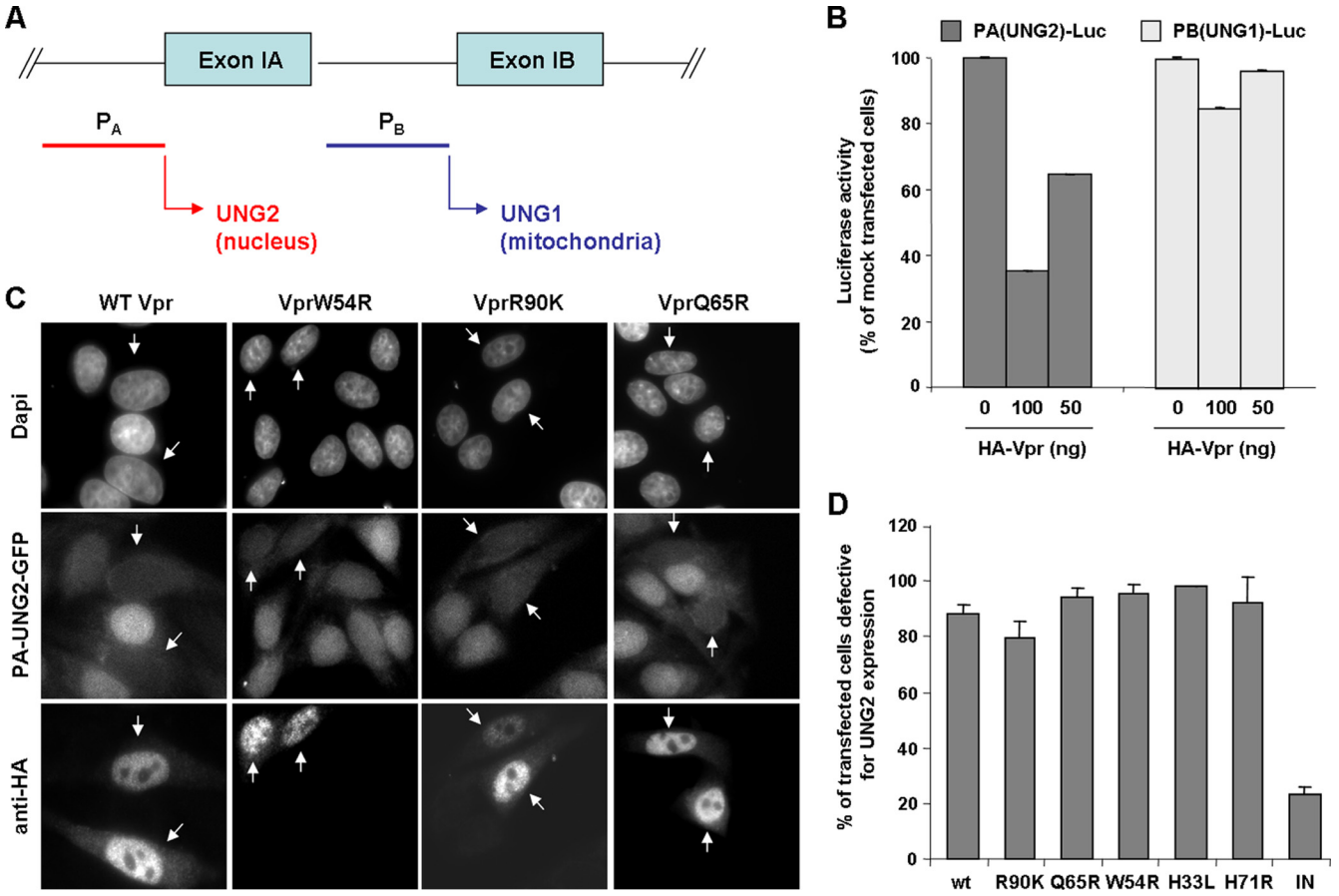

FIG. 5. Impact of Vpr on UNG1 and UNG2 promoters. (A) Schematic representation of the structural organization of the promoter region of the human *ung* gene according to Nilsen et al. (18). The PA promoter is used for expression of the nuclear UNG2 isoform, whereas the PB promoter is used for expression of the mitochondrial UNG1 isoform. (B) UNG1 and UNG2 promoter activity upon Vpr expression. All promoter constructs were as already described (8, 18). HeLa cells were thus cotransfected with reporter constructs in which expression of firefly luciferase was driven by either PA [PA(UNG2)-Luc] or PB [PB(UNG1)-Luc] promoters in combination with the pRL-TK vector encoding *Renilla* luciferase and increasing amounts (50 and 100 ng) of the HA-Vpr-expressing vector. At 48 h after transfection, cells were lysed and luciferase activity was measured, using a dual luciferase assay kit (Promega) in a luminometer (Berthold Instruments). The promoter activity was then determined as a ratio between firefly and *Renilla* luciferase measurement in cell extracts. (C) HeLa cells stably expressing the UNG2-GFP fusion under the control of the PA *UNG2* promoter were transfected with vectors for expression of wild-type or mutated HA-Vpr proteins. After 24 h, the cells were fixed, permeabilized, and subsequently stained with anti-HA (lower panels). Cells were then analyzed by epifluorescence microscopy as described in Fig. 3A. (D) The number of cells with a reduced UNG2-GFP signal was quantified over 100 cells expressing wild-type or mutated HA-Vpr, or HA-IN; the results are expressed as the percentage of transfected cells showing defective UNG2 staining. Values are the means of three independent experiments. WT, wild type.

Vpr-induced reduction of UNG2 is related to a transcriptional regulation. Our analyses suggested that Vpr affected the level of UNG2 without apparent effect on the UNG1 mitochondrial isoform. Both proteins are encoded by the same gene but are generated by the use of different promoters (Fig. 5A) and alternative splicing (8, 18). While the PA promoter is used for generation of UNG2, PB is responsible of UNG1 expression. Since it was reported that Vpr is able to regulate the transcription of host cell genes through binding to transcription factors (9, 27), we decided to explore whether Vpr might rather act at the transcriptional level to regulate UNG2 expression. HeLa cells were thus transfected with reporter constructs in which expression of firefly luciferase was driven by either PA [PA(UNG2)-Luc] or PB [PB(UNG1)-Luc] promoters in combination with the pRL-TK vector encoding *Renilla* luciferase and the HA-Vpr-expressing vector (19). Promoter activity was determined 48 h after transfection as a ratio between firefly and *Renilla* luciferase measurement in cell extracts. Using the PA(UNG2)-Luc construct, a dose-dependent decrease in the luciferase activity was detected in Vpr-expressing cells (Fig. 5B). In contrast, similar experiments performed with the PB(UNG1)-Luc construct did not show significant modification in the Luciferase activity ratio. In addition, we checked that Vpr expression also decreased the level of UNG2 using HeLa cells stably expressing the UNG2-GFP fusion under the control of the PA *UNG2* promoter (Fig. 5C). As expected, all of the Vpr mutants described above, deficient for UNG2 binding, G_2 arrest, or both, efficiently decreased the level of UNG2-GFP expression when expressed in this cell line (Fig. 5C and D). These results suggest that UNG2 is specifically downregulated by the Vpr protein at the transcriptional level.

By monitoring the level of endogenous UNG2, we confirm that the expression of Vpr significantly reduced the level of UNG2 found in HIV-1-infected cells. Surprisingly, this effect was reverted neither by treatment with proteasome inhibitors, nor by the well-characterized VprW54R variant that fails to interact with UNG2 (23). In contrast to published reports, these results indicate that the Vpr-mediated regulation of UNG2 level is not related to the targeting of UNG2 to proteasomal degradation through direct interaction with Vpr (22). These differences are likely related to the experimental systems used in previous studies in which analyses were performed in transfected cells overexpressing exogenous UNG2 protein under cytomegalovirus promoters. Interestingly, our data also indicate that the downregulation of UNG2 is independent of the Vpr-mediated G_2 arrest, since it was observed in cells expressing G_2 arrest-deficient mutants, including the VprQ65R mutant that is deficient for interaction with the DCAF1 subunit of the Cul4a/DDB1 E3 ligase. These results argue against a major role of the proteasome machinery in the Vpr-induced UNG2 downregulation. Although we cannot exclude that proteasomal degradation is involved in the Vpr-induced downregulation of UNG2, our data using Vpr mutants deficient for binding to UNG2, for G_2 arrest activity, or both (H33L and H71R) strongly suggest that the reduction of UNG2 observed in Vpr-expressing cells is not related to Vpr binding or Vpr manipulation of the cell cycle. Finally, we provided evidence that Vpr negatively regulates the PA(UNG2) promoter, suggesting that the decrease of the UNG2 protein level is rather due to a transcriptional effect of Vpr on UNG2 expression. Interestingly, the PA and PB promoters are regulated by distinct specific positive and negative transcription factors, and further analyses should help to understand how Vpr specifically acts on PA(UNG2).

Since we and others have proposed that UNG2 was required to modulate the virus mutation rate and for efficient virus replication in macrophages (3, 15), our present observations suggest that UNG2 may participate in the virus life cycle via two distinct Vpr-mediated mechanisms: while the first mechanism is related to Vpr binding and leads to the incorporation of UNG2 into virions for contributing to the fidelity of the reverse transcription upon infection, the second mechanism is independent of Vpr binding but is related to a negative transcriptional effect on UNG2 expression in virus producing cells. Additional studies are thus required for elucidation of the respective role of these apparent contradictory effects and to understand the real contribution of the functional and physical interactions between Vpr and UNG2 during HIV-1 infection.

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