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# Molecular Insight into How HIV-1 Vpr Protein Impairs Cell Growth through Two Genetically Distinct Pathways\*<sup>§</sup>

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Vpr, a small HIV auxiliary protein, hijacks the CUL4 ubiquitin ligase through DCAF1 to inactivate an unknown cellular target, leading to cell cycle arrest at the G<sub>2</sub> phase and cell death. Here we first sought to delineate the Vpr determinants involved in the binding to DCAF1 and to the target. On the one hand, the three  $\alpha$ -helices of Vpr are necessary and sufficient for binding to DCAF1; on the other hand, nonlinear determinants in Vpr are required for binding to the target, as shown by using protein chimeras. We also underscore that a SRIG motif conserved in the C-terminal tail of Vpr proteins from HIV-1/SIVcpz and HIV-2/SIVsmm lineages is critical for G<sub>2</sub> arrest. Our results suggest that this motif may be predictive of the ability of Vpr proteins from other SIV lineages to mediate G<sub>2</sub> arrest. We took advantage of the characterization of a subset of G<sub>2</sub> arrest-defective, but DCAF1 binding-proficient mutants, to investigate whether Vpr interferes with cell viability independently of its ability to induce G<sub>2</sub> arrest. These mutants inhibited cell colony formation in HeLa cells and are cytotoxic in lymphocytes, unmasking a G<sub>2</sub> arrest-independent cytopathic effect of Vpr. Furthermore these mutants do not block cell cycle progression at the G<sub>1</sub> or S phases but trigger apoptosis through caspase 3. Disruption of DCAF1 binding restored efficiency of colony formation. However, DCAF1 binding *per se* is not sufficient to confer cytopathicity. These data support a model in which Vpr recruits DCAF1 to induce the degradation of two host proteins independently required for proper cell growth.

Primate lentiviruses such as HIV type 1 and type 2, the causative agents of human AIDS, are complex retroviruses. During

their evolution they have acquired a set of auxiliary proteins that have no counterpart in gamma retroviruses such as murine leukemia virus. There is increasing evidence indicating that, in the setting of natural infection, auxiliary proteins play an important role in the relationship between HIV and its host cells by adjusting the cellular context for optimal viral growth (1).

Vpr remains the most enigmatic of the auxiliary proteins from HIVs. So far the functional importance of Vpr is mostly argued for by genetic evidence; the conservation of the Vpr gene between HIV-1 and HIV-2/SIVsm (2) and the reversions toward wt Vpr expression in macaques experimentally infected with SIV mutated in the Vpr gene (3). In primary macrophages, which are nondividing cells, deletion of Vpr has been reported to slightly reduce HIV-1 infection efficiency in several studies (4–7). In contrast, lack of Vpr does not affect viral infection of dividing cells in a measurable way. Somewhat ironically, the most widely admitted property of Vpr, *i.e.* its ability to specifically block the cell cycle progression at the G<sub>2</sub>/M transition, can by essence only be observed in such cells (8). This property is highly conserved among a series of primate lentiviruses but is not shared by Vpr from SIVagm (African green monkey) (9–11), at least in human cells. Several studies have reported that Vpr-mediated G<sub>2</sub> arrest depends on the DNA damage checkpoint pathway, which involves the ATR (ataxia telangiectasia-mutated and Rad3-related) kinase and the phosphorylation of Chk1 (12–17). This pathway is suspected to result from an S phase-dependent mechanism and the association of Vpr with chromatin (13, 16, 18, 19). In 2007, we and others identified DCAF1/VprBP as an essential host factor in the ability of Vpr to promote G<sub>2</sub> arrest (20–26). DCAF1 is an adaptor subunit of DDB1, a core component of Cullin 4 (CUL4)-based ubiquitin ligases and of the less well characterized EDD ubiquitin ligase (27, 28). Selection of proteins by ubiquitin ligases is a prerequisite for their subsequent proteasome-mediated degradation (29). Vpr has recently been shown to mediate specifically Lys-48-linked polyubiquitination of target cellular proteins (30). Therefore, the unifying model is that Vpr uses the CUL4<sup>DCAF1</sup> ubiquitin ligase to promote the inactivation of a so far unidentified protein target (hereafter referred to as the G<sub>2</sub> target) that is required for entry into mitosis (20–26). As a result, cell cycle progression

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is arrested at the G<sub>2</sub>/M transition, leading eventually to cell death through an apoptotic pathway (for review, see Ref. 8). The use of the DCAF1 adaptor by Vpr to promote inactivation/degradation of a specific host protein argues that in some settings this G<sub>2</sub> target represents a host barrier for optimal viral growth. In this respect, the fine dissection of Vpr properties remains of great interest to obtain further clues about its function and to help decipher the nature of its putative target. To date, the Vpr determinants involved in the ability of Vpr to recruit DCAF1 and the G<sub>2</sub> target are not fully characterized. Vpr contains a hydrophobic core of three  $\alpha$ -helices surrounded by N- and C-terminal flexible domains (31). Mutations all along the molecule have been described to alter the ability of Vpr to mediate G<sub>2</sub> arrest, in particular those located in the C-terminal tail of the protein, which has been proposed to be involved in the binding to the G<sub>2</sub> target (18, 21, 23). Point mutations in the third  $\alpha$ -helix abrogate binding to DCAF1, but whether this helix constitutes a binding site for DCAF1 is unclear (20, 23–25).

In the present study we first sought to delineate the Vpr determinants required for the recruitment of both DCAF1 and the G<sub>2</sub> target. To characterize the G<sub>2</sub> target binding module in Vpr, we isolated a series of Vpr mutants, which bind DCAF1 but do not arrest cell cycle progression. The corresponding mutations lie in a SRIG motif highly conserved in the C-terminal tail of Vpr proteins from HIV and SIV lineages. Strikingly, a chimera between a DCAF1-binding module, unrelated to HIV-1 Vpr, and the C-terminal tail of HIV-1 Vpr is not able to mimic the G<sub>2</sub> arrest activity of wt HIV-1 Vpr, suggesting that Vpr recruits its G<sub>2</sub> target through a nonlinear physical determinant. In agreement with this hypothesis, mutations in the core helical domain of Vpr also impair G<sub>2</sub> arrest while preserving DCAF1 binding. We then took advantage of the characterization of a subset of G<sub>2</sub> arrest-defective but DCAF1 binding-proficient mutants to investigate whether Vpr interferes with cell viability independently of its ability to induce G<sub>2</sub> arrest. We underscore a Vpr-mediated pathway that leads to cell death independently of any cell cycle block. As is the case for the G<sub>2</sub> arrest activity, recruitment of DCAF1 appears necessary but not sufficient to trigger this cell response. These results raise the hypothesis that Vpr may inactivate two distinct host factors required for normal cell growth (hereafter referred to as the G<sub>2</sub> target and the cytotoxicity target).

## EXPERIMENTAL PROCEDURES

**Plasmid Constructs**—The pAS1B vector encoding HA-tagged Vpr from HIV-1 LAI, Vpx from SIVsmPBj, and Vpr from SIVagm has been previously described (23, 33). Site-directed mutagenesis was performed using the QuikChange kit from Stratagene. The genes corresponding to SIVrcm, SIVmnd-2, and the chimera between HIV-1 Vpr and SIVagm Vpr or SIVsmPBj Vpx were synthesized by GeneCust Europe after codon optimization for expression in human cells and then inserted into the pAS1B vector. For the clonogenic assays, Vpr from HIV-1 LAI, its mutants, Vpr from SIVagm and Vpx from SIVsmPBj, were inserted into pCT152, an Epstein-Barr virus (EBV)-based episomal vector described in Sitterlin *et al.*

(34). All sequences were checked by DNA sequencing. The plasmid encoding the DCAF1 isoform 1 fused to three FLAG tags at the N terminus has been previously described (35). The internal membrane-anchored GFP was expressed from the pBabe/GEM2 vector (36).

**Yeast Methods**—Two-hybrid experiments were performed in the L40 yeast strain as previously described (23).

**Cell Culture and Transfection Procedures**—HeLa and 293T cells were maintained in DMEM, and Jurkat cells were maintained in RPMI; both were supplemented with glutamine and 10% fetal calf serum. Plasmid transfections were performed using FuGENE 6 Transfection reagent (Roche Applied Science).

**Cell Cycle Analysis**— $5 \times 10^5$  HeLa cells were plated into 6-cm dishes 24 h before transfection. The cells were transfected with 1  $\mu$ g of pAS1B-Vpr (wt or mutated) in combination with 0.1  $\mu$ g of pBabe/GEM2 as an internal transfection marker. Twenty-four hours later, the cells were harvested and plated into 10-cm dishes and grown for one more day. The cells were then detached (manually) and fixed in 70% ethanol. After treatment for 30 min at 37 °C with 0.2 mg/ml RNase A and 50  $\mu$ g/ml propidium iodide in buffer H (20 mM HEPES, 160 mM NaCl, 1 mM EGTA), cells expressing the cotransfected GFP were analyzed for their DNA content using a Cytomics FC500 cell analyzer (Beckman Coulter). At least 10,000 GFP-positive cells were analyzed for their distribution in the different phases of cell cycle.

**Immunoprecipitation Procedure**—Cells grown in 10-cm dishes were lysed in 300  $\mu$ l of SD buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100) containing an anti-protease mixture (Sigma). Cell lysates were clarified by centrifugation and incubated with anti-FLAG beads (EZview™ Red ANTI-FLAG® M2 Affinity Gel, Sigma) for 2 h at 4 °C. After two washes in SD buffer, immunoprecipitated proteins were recovered by elution with FLAG peptide (Sigma) for 1 h at 4 °C.

**Western Blot Procedure and Antibodies**—Cells were lysed in 300  $\mu$ l of M-PER buffer (Pierce) containing 150 mM NaCl and an anti-protease mixture (Sigma), and protein extracts were separated by SDS-PAGE electrophoresis. After transfer onto PVDF membranes, proteins were revealed by immunoblot analysis using a chemiluminescent procedure (CDPStar®, Applied Biosystems). Signals were acquired by a LAS 3000 apparatus (Fujifilm) for further quantification using the Multigauge software (Fujifilm). Monoclonal antibody directed against the HA (16B12) tag was purchased from Covance Research Products, anti-GFP monoclonal antibody was obtained from Roche Applied Science, anti-FLAG M2 monoclonal antibody was purchased from Sigma, and anti-caspase 3 rabbit polyclonal antibody was obtained from Cell Signaling.

**Clonogenic Assay**—HeLa cells ( $2 \times 10^5$ ) were plated in 12-well plates 24 h before transfection. Cells were transfected in duplicate wells with 0.5  $\mu$ g of pCT152 episomal constructs expressing viral proteins. Cells were harvested 48 h post-transfection, and 10% of the cells were plated into 10-cm dishes containing DMEM supplemented with 0.5 mg/ml hygromycin (Invitrogen) and grown for 15 days. Cell colonies were then washed with PBS, fixed for 15 min in 100% ethanol at 4 °C, and stained with 10% Giemsa (Sigma) for 15 min.

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**DNA Synthesis Assay**—DNA synthesis was analyzed by measuring the incorporation of EdU,<sup>7</sup> a nucleoside analog of thymidine, using the Click-iT<sup>®</sup> EdU Alexa Fluor<sup>®</sup> 647 Flow Cytometry Assay kit (Invitrogen). Briefly, cells were incubated 1 h with 10  $\mu$ M EdU, fixed, and processed following the manufacturer's instructions. Cytometry analysis was carried out using a Cytomics FC500 cell analyzer (Beckman Coulter).

**Pseudoparticles Production**—293T cells ( $4 \times 10^6$  cells) were cotransfected with HIV-1 minimal packaging vector pCMV $\Delta$ 8.91 (37) along with two plasmids coding for vesicular stomatitis virus glycoprotein and HA-tagged Vpr in a ratio of 5:1:5. The culture supernatants were collected 48 h after transfection and filtered through 0.45- $\mu$ m pore filters. The viral particles were then concentrated in 10% polyethylene glycol 6000 (PEG-6000) (Sigma) containing 300 mM NaCl and titrated by quantification of HIV-1 capsid p24 using an enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix Corp.).

**Annexin-FITC Labeling**—Jurkat cells incubated with pseudoparticles were harvested and washed in ice-cold PBS. The cells were then labeled with annexin-FITC (FITC Annexin V/Dead Cell Apoptosis Kit V13242 from Invitrogen) in annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>, pH 7.4) for 15 min at room temperature. Cells were further analyzed using a Cytomics FC500 cell apparatus (Beckman Coulter).

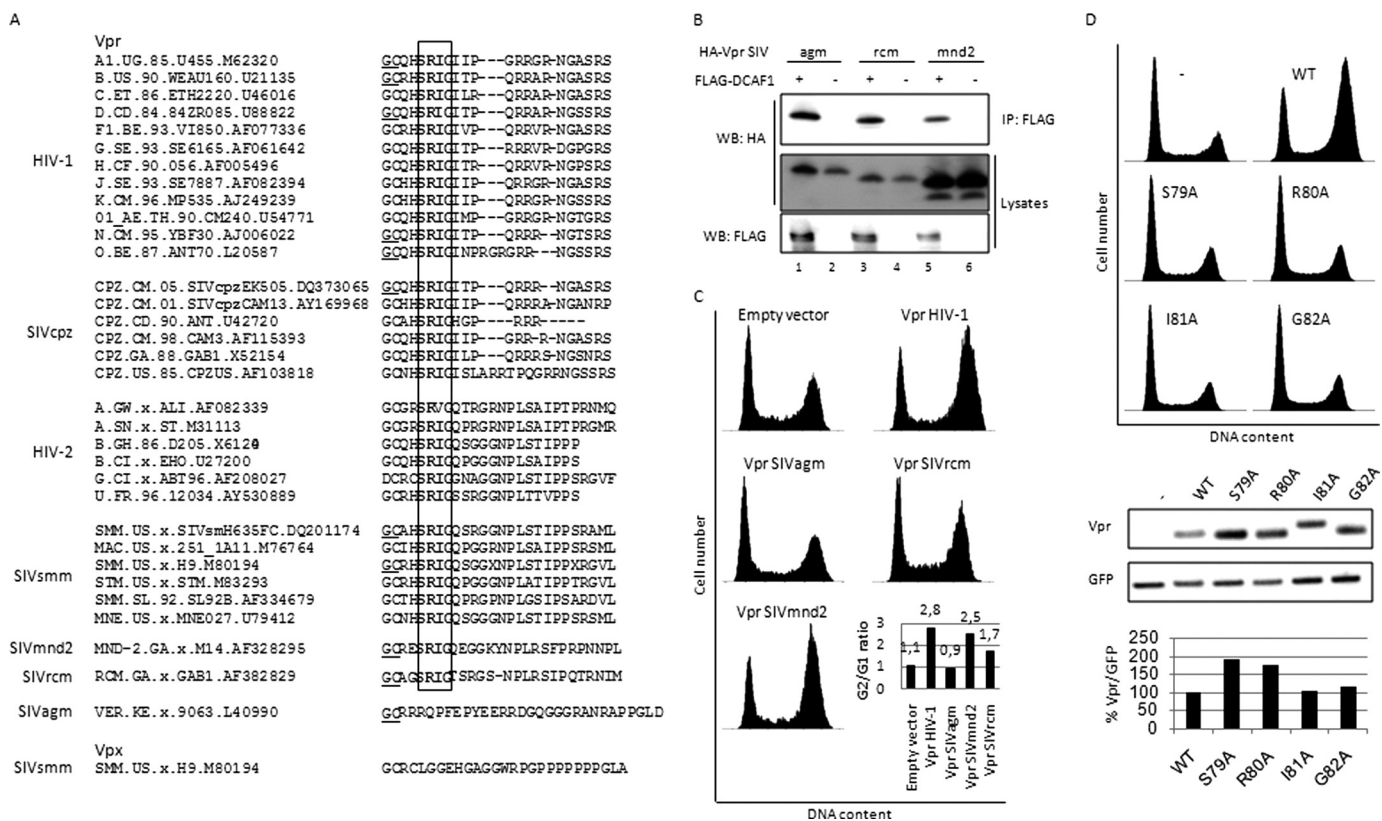
## RESULTS

**A Highly Conserved SRIG Motif in the C-terminal Tail of Vpr Is Critical for Vpr-mediated G<sub>2</sub> Arrest**—We previously showed that removal of the C-terminal tail of Vpr, which preserves DCAF1 binding, leads to a *trans*-dominant-negative phenotype as the truncated mutant fails to induce G<sub>2</sub> arrest when expressed in *cis* but inhibits the cell cycle arrest activity of the wt protein when expressed in *trans* (23). Similar data were reported by DeHart *et al.* (21) using the R80A mutation, which maps to the C-terminal tail. This revealed the importance of the latter region in the recruitment of the G<sub>2</sub> target. To gain deeper insight into the Vpr determinants required to recruit the G<sub>2</sub> target, we took advantage of the previous knowledge that the cell cycle arrest activity is conserved by the Vpr orthologs of HIV-1/SIVcpz and HIV-2/SIVsmm lentiviral lineages despite the proteins being quite divergent in their C-terminal regions. Thus, the C terminus of HIV-1 Vpr comprises a functionally important stretch of positively charged arginine residues (Arg-87, -88, -90) (38), but these residues are not conserved throughout the different lentiviral lineages. Close inspection of the multiple alignment shown in Fig. 1A revealed the striking conservation of a SRIG motif across the Vpr orthologs from these lentiviral lineages. The motif was absent in the Vpr protein from SIVagm, previously found to be G<sub>2</sub> arrest-defective in human cells (10, 11), and in the Vpx protein found in the HIV-2/SIVsmm lineage, which is genetically related but functionally non-redundant with Vpr (9, 10). We thus wondered whether the presence of a SRIG motif might be predictive of the ability of Vpr proteins to induce

G<sub>2</sub> arrest. The SRIG motif was present in the non-previously characterized Vpr proteins from two SIV lentiviral lineages, SIVmnd-2 (Mandrill) and SIVrcm (Red Capped Mangabey). This prompted us to investigate the ability of Vpr proteins from these viruses to mediate G<sub>2</sub> arrest. Both proteins were able to bind DCAF1 in co-immunoprecipitation assays (Fig. 1B) and to arrest cell cycle progression (Fig. 1C), in contrast to Vpr from SIVagm, which readily co-immunoprecipitates DCAF1 (Fig. 1B) but does not trigger G<sub>2</sub> arrest (Fig. 1C). We next undertook a mutational analysis of the conserved SRIG motif using the HIV-1 Vpr as a prototype. As expected from previous studies (39, 40), substitution of the Ser-79 and Arg-80 residues for alanine abrogated cell cycle activity. Similarly, the I81A and G82A mutants failed to promote G<sub>2</sub> arrest (Fig. 1D, *top panel*). The expression of the SRIG mutants was verified by Western blot to ensure that loss-of-function was not merely due to their decreased stabilities (Fig. 1D, *bottom panel*).

**Binding of Vpr to the G<sub>2</sub> Target Occurs through a Nonlinear Physical Determinant**—We next investigated whether the Vpr C-terminal region was sufficient to recruit the G<sub>2</sub> target in the context of a DCAF1 binding molecule. To examine this possibility we first delineated the minimal DCAF1-binding region of Vpr using a yeast two-hybrid assay with various Vpr truncated mutants. The three helical regions of Vpr from residues 17–74 were necessary and sufficient to confer binding to DCAF1 (Fig. 2, A and B, for a diagram of Vpr structure). The structure of Vpr is predicted to be shared by the genetically related Vpx protein, which is specific to the HIV-2/SIVsmm lineage. Vpx does not trigger G<sub>2</sub> arrest but also subverts the host CUL4<sup>DCAF1</sup> ubiquitin ligase to inactivate an evolutionary conserved factor, which impairs accumulation of viral reverse transcripts in macrophages (33, 41, 42). As expected from the mapping of the DCAF1 binding region in Vpr, the DCAF1 binding region of Vpx was also confined to the predicted helical regions of the protein (Fig. 2, A and B). As a DCAF1 binding module, we thus selected the N-terminal region of Vpx with its three helices. The chimeric molecule, which consists of this module fused to the C-terminal tail of Vpr (X-R) (Fig. 2B), conserved the ability to bind DCAF1 (Fig. 2C) but failed to promote G<sub>2</sub> arrest (Fig. 2D). We did not exclude that the inability of X-R to induce G<sub>2</sub> arrest was due to the lower expression of the chimeric protein compared with HIV-1 Vpr (Fig. 2C, compare *lanes 3 and 4* and *lanes 5 and 6, middle panel*). Thus, we constructed a second chimeric protein consisting of the N terminus of Vpr from SIVagm, fused to the C-terminal tail of HIV-1 Vpr (Ragm-R). This chimera was as well expressed as wt HIV-1 Vpr (Fig. 2C, compare *lanes 5 and 6* to *lanes 9 and 10*) and could still recruit DCAF1 (Fig. 2C, *lane 9*) but was also defective for G<sub>2</sub> arrest (Fig. 2D). Therefore, the C-terminal tail of Vpr was not sufficient to recapitulate the original function of the protein when fused to the DCAF1 binding core of either SIVsmm Vpx or SIVagm Vpr. These observations led us to predict the existence of mutations in Vpr that would meet the following criteria: (i) inactivation of the G<sub>2</sub> arrest activity, (ii) conservation of DCAF1 binding, and (iii) location outside of the C-terminal tail. The phenotypes of the K27M and Y50A mutations, which lie in helix  $\alpha$ 1 and in the linker region between  $\alpha$ 2 and

<sup>7</sup> The abbreviations used are: EdU, 5-ethynyl-2'-deoxyuridine; EBV, Epstein-Barr virus; UNG2, uracil-DNA glycosylase-2; VLP, virus-like particles.



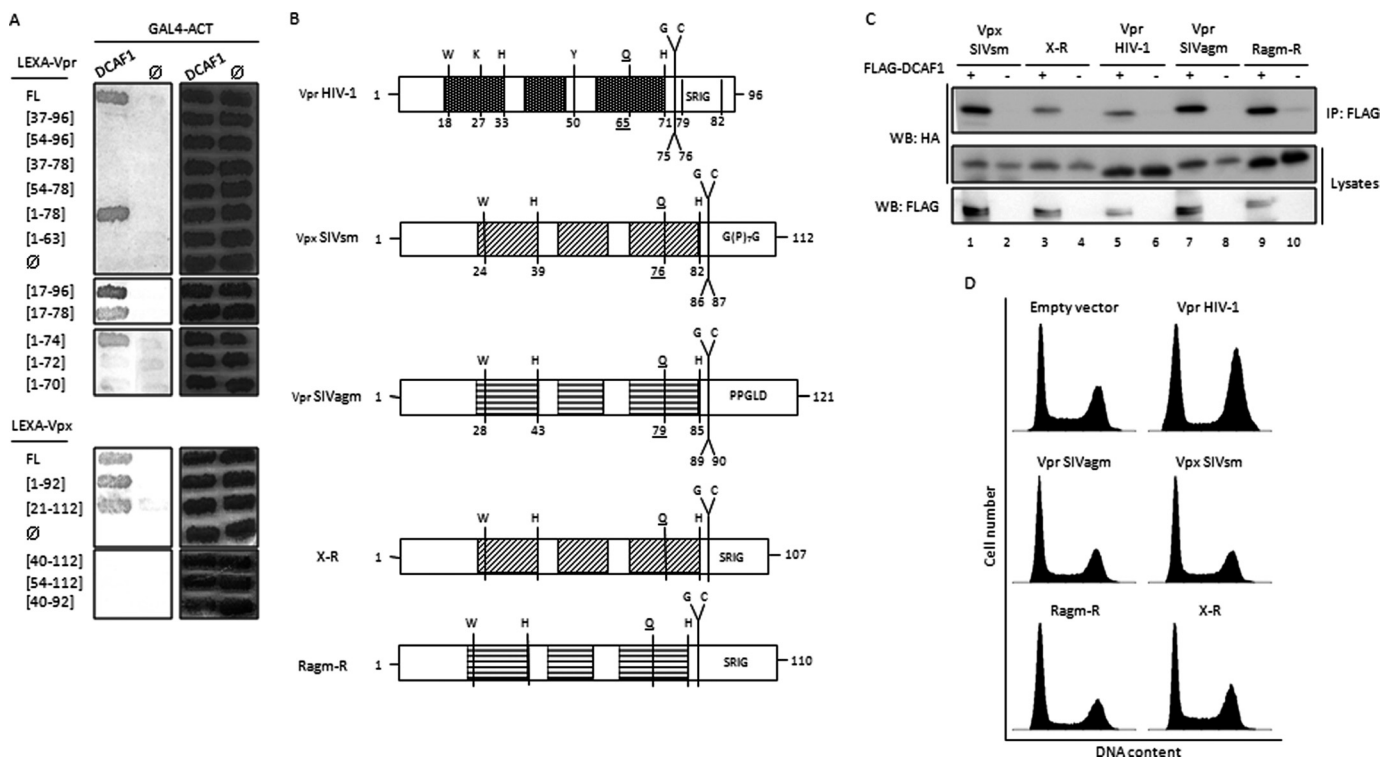
**FIGURE 1. The C-terminal tail of Vpr shows a highly conserved SRIG motif, which is critical for cell cycle arrest activity.** *A*, identification of a highly conserved SRIG motif in the C-terminal tail of Vpr from HIV-1/SIVcpz and HIV-2/SIVsmm/SIVmnd2/SIVrcm is shown. The Vpr and Vpx proteins with the indicated accession numbers were aligned from the end of the predicted third  $\alpha$ -helix as defined by the underlined invariant GC di-amino acid motif to their C termini. The lentiviral origin of the proteins is indicated on the left side. The conserved SRIG motif is boxed. *B*, the Vpr proteins from SIVmnd-2 and SIVrcm both bind DCAF1. Cell lysates were prepared 48 h post-transfection and subjected to immunoprecipitation using anti-FLAG antibodies covalently coupled to Sepharose beads. After extensive washing, bound proteins were eluted from beads with the FLAG peptide. Immunoprecipitates (IP) and crude cell lysates (Lysates) were analyzed by Western blotting using the indicated antibodies. *C*, SIVmnd-2 Vpr and SIVrcm Vpr arrest the cell cycle similarly to HIV-1 Vpr. HeLa cells were transfected with vectors expressing the indicated HA-tagged proteins along with a vector expressing the GFP protein. Cells were harvested 48 h post-transfection. After fixation and propidium iodide staining, the cells were analyzed by flow cytometry to monitor the DNA content of the GFP-positive population. The  $G_2/G_1$  ratio is indicated in the bar graph. *D*, each residue of the SRIG motif is critical for the cell cycle arrest activity of HIV-1 Vpr. Alanine substitutions of each residue of the SRIG motif were introduced into the Vpr protein from HIV-1 LAI by site-directed mutagenesis. The Vpr-mutated proteins were expressed into HeLa cells to monitor their effect on cell cycle progression in comparison to wt Vpr. Cell cycle analysis was performed as described above (top). A fraction of cells was lysed, and the indicated HA-tagged Vpr proteins as well as the co-transfected GFP protein were revealed by Western blot analysis. Quantification of the signals enabled to deduce the Vpr to GFP protein expression ratio that is represented on the bar graph (bottom).

$\alpha$ 3 helices, respectively, fully confirmed this prediction (Fig. 3 for VprK27M, supplemental Fig. Fig. 1 for VprY50A). As previously shown for Vpr S79A (20, 23), VprK27M was able to recruit DCAF1 (Fig. 3A, lane 6) but did not trigger  $G_2$  arrest (Fig. 3B). The VprK27M/S79A double mutant behaved as the single mutants, whereas the previously characterized DCAF1 binding-deficient Q65R mutant did not arrest the cell cycle. Of note, the K27M mutant has been previously reported as a  $G_2$ -arrest inactive mutant (7), although its DCAF1 binding status was to date unknown.

**$G_2$  Arrest-defective Vpr Mutants Unmask a Second Cytopathic Activity of Vpr, Which Correlates with Its Ability to Recruit the  $CUL4^{DCAF1}$  Ubiquitin Ligase**—The apoptotic cell death resulting from the prolonged  $G_2$  arrest caused by Vpr is widely admitted in the field (for review, see Ref. 8). A  $G_2$  arrest-independent cell death induced by HIV-1 Vpr has been observed by two groups (43–45), in contrast to previous studies (11, 46, 47). In their hands,  $G_2$  arrest-defective Vpr mutants remain cytopathic. Moreover, they both showed that this second cytopathic activity of Vpr results from a cell cycle arrest at

the  $G_1$  phase (43, 44). However, the DCAF1 binding status of their mutants was unknown. Thus, we revisited this question using the set of mutants we characterized. We developed a colony-forming assay based on the expression of Vpr from an EBV-derived episomal vector. This system combines several advantages, among which are the episomal maintenance of the transgene that precludes the confounding effects of the host genome insertion sites on transgene expression and the selection of transfected cells using the built-in hygromycin resistance gene. As shown in Fig. 4, A and B, expression of wt HIV-1 Vpr dramatically reduced the ability of cells to form hygromycin-resistant colonies within 15 days (less than 1% of the control). The K27M and S79A Vpr mutants also strongly reduced colony formation (less than 5 and 10% of the control, respectively). The K27M/S79A double mutant showed the same phenotype as the single mutants, ruling out the hypothesis that cytotoxicity may result from a residual ability of the single mutants to inactivate the  $G_2$  target (Fig. 4C). Therefore,  $G_2$  arrest-defective Vpr mutants conserve the ability to induce cytotoxicity.

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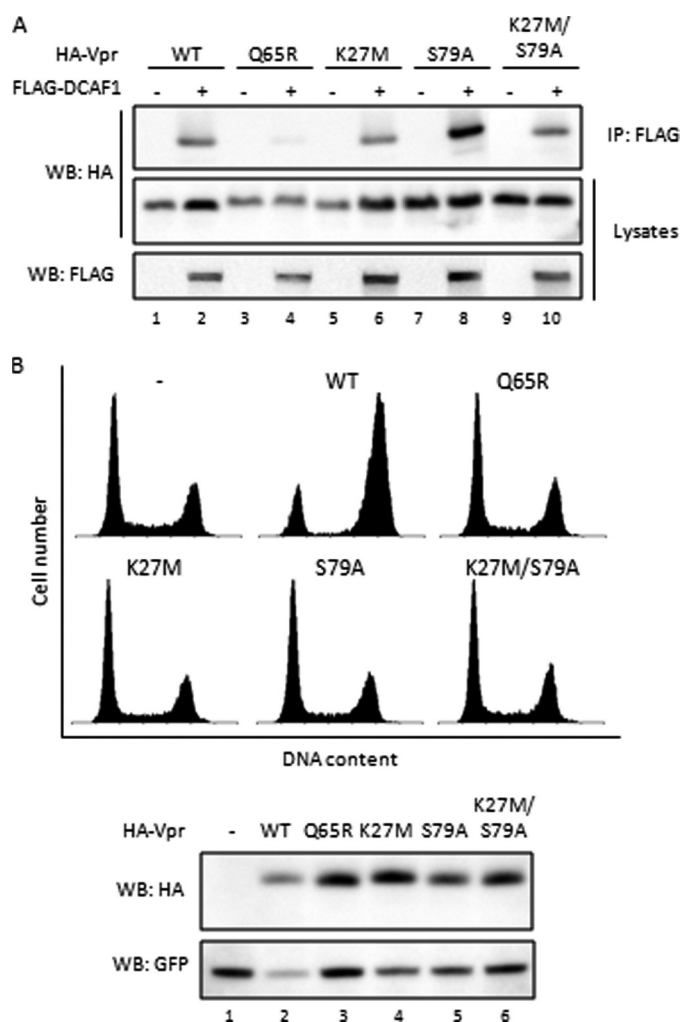
**FIGURE 2. Chimeric proteins between a DCAF1 binding module and the C-terminal tail of HIV-1 Vpr are defective for  $G_2$  arrest activity.** *A*, the three- $\alpha$ -helices core domain of HIV-1 Vpr and SIVsmm Vpx is necessary and sufficient to bind DCAF1. The indicated hybrid proteins were co-expressed in the L40 yeast reporter strain, and the yeast transformants were analyzed for induction of  $\beta$ -galactosidase (left panel). As a control, the right panel shows the normal growth in permissive medium of the corresponding yeast transformants. *B*, the structure of the HIV-1 Vpr, SIVsmm Vpx, and SIVagm Vpr proteins and of their chimeric X-R and Ragm-R derivatives is shown. The viral proteins are depicted as rectangles. Their predicted three  $\alpha$ -helices, hereafter denoted  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ , are represented by dotted and hatched boxes. Shown are the positions of several residues fully conserved between the three proteins including the Tyr and His residues in  $\alpha 1$ , the Gln and His residues in  $\alpha 3$ , and the GC di-amino acid at the boundary between  $\alpha 3$  and the C-terminal tail. The Gln residue, which is critical for DCAF1 binding, is underlined. The SRIG motif found in the C-terminal tail of HIV-1 Vpr as well as the PPGLD motif found in the C-terminal tail of SIVagm Vpr and a conserved G-P rich motif found in the C-terminal tail of SIVsmm Vpx are indicated. *C*, X-R and Ragm-R chimera both bind DCAF1. Co-immunoprecipitation (IP) experiments were performed as described in Fig. 1B. *D*, fusion of the C-terminal tail of HIV-1 Vpr to the DCAF1-binding region of SIVsmm Vpx or SIVagm Vpr fails to restore the  $G_2$  arrest activity of wt Vpr. HeLa cells expressing the indicated constructs were analyzed for their cell cycle profile as described in Fig. 1C.

Next, we wondered whether this  $G_2$  arrest-independent cytopathicity also relies on the recruitment of the CUL4<sup>DCAF1</sup> ubiquitin ligase. The Q65R mutation, which abrogates DCAF1 binding, restored efficient colony formation either when borne as a single mutation (Fig. 4, *A* and *B*, more than 70% of the control) or in combination with the K27M or the S79A mutations (Fig. 4C). We conclude that Vpr from HIV-1 exhibits a  $G_2$  arrest-independent but DCAF1 binding-dependent cytopathic activity. These data raised the possibility that the DCAF1 binding activity of Vpr *per se* was deleterious for cell growth. However, Vpx from SIVsmm and Vpr from SIVagm, which both bind DCAF1 with the same efficiency as HIV-1 Vpr (Fig. 5A), did not reduce colony formation efficiency (Fig. 5B). Therefore, DCAF1 binding is not sufficient to trigger  $G_2$  arrest-independent cytotoxicity.

**Cell Death Triggered by  $G_2$  Arrest-defective Vpr Mutants Is Independent of Vpr Binding to Uracil-DNA Glycosylase-2 (UNG2)**—UNG2, which excises uracil bases in single-stranded and double-stranded DNA in the base excision repair pathway, has been proposed as a Vpr-inactivated host target (48, 49). In addition, Vpr-mediated  $G_2$  arrest does not depend on Vpr binding to UNG2 (50). To test whether UNG2 recruitment by Vpr might mediate its  $G_2$  arrest-independent cytopathic effect, we introduced the previously described W54R muta-

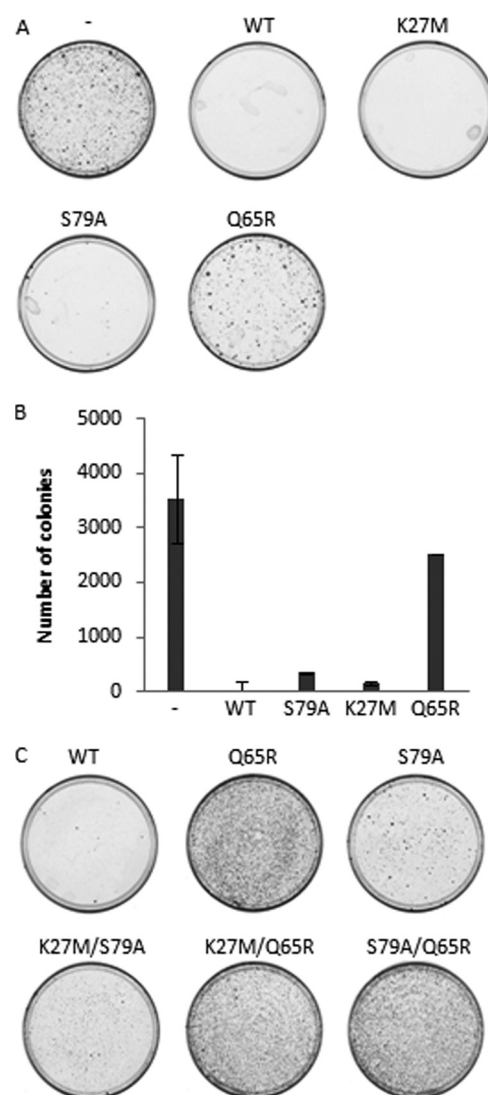
tion (50) that impairs Vpr binding to UNG2 into the Vpr S79A mutant. The double mutant Vpr W54R/S79A was still able to bind DCAF1 (Fig. 6A) and to trigger cell death (Fig. 6B), indicating that Vpr-UNG2 interaction is not involved in Vpr-mediated cytotoxicity.

**Cell Death Triggered by  $G_2$  Arrest-defective Vpr Mutants Occurs through Apoptosis and Independently of Cell Cycle Progression**—To gain insight into the mechanism underlying this second cytopathic activity of Vpr, we carried out large-scale transfections in HeLa cells using the aforementioned EBV-derived constructs. Two days after transfection, the cells were plated into hygromycin-containing medium, and their viability was inspected on a daily basis. As expected, cells expressing wt HIV-1 Vpr or cells transfected with an empty vector died massively within 3 days post-selection. At the same time point, cells expressing either VprQ65R or wt SIVsmm Vpx as well as cells expressing VprS79A and VprK27M were still growing (data not shown). However, by 5 days post-selection, VprS79A- and VprK27M-expressing cells exhibited dramatic morphologic changes and stopped growing, whereas VprQ65R and wt SIVsmm Vpx expressing cells remained healthy. Flow cytometry analysis of the cell DNA content was performed on the total population to include cells with a DNA content inferior to 2N (sub- $G_1$  cells), indicative of an apoptotic cell death. From



**FIGURE 3. Vpr mutations lying outside of the C-terminal tail abrogate  $G_2$  arrest activity while preserving DCAF1 binding function.** *A*, the K27M and K27M/S79A Vpr mutants efficiently co-immunoprecipitate (IP) with DCAF1. Constructs expressing the indicated proteins were transfected into HeLa cells. Cell lysates were prepared 48 h post-transfection and subjected to immunoprecipitation as described in Fig. 1*B*. Note that the VprQ65R mutant is unable to co-immunoprecipitate with DCAF1 consistent with our previously published results (23). *WB*, immunoblot. *B*, the K27M Vpr mutant fails to promote  $G_2$  arrest. HeLa cells expressing the indicated proteins were analyzed for their cell cycle profiles and for protein expression as described in Fig. 1*C*.

3–5 days post-selection, an increasing fraction of cells expressing VprS79A or VprK27M showed a less than 2N content in DNA (Fig. 7*A*). Proteolytic activation of caspase 3, a marker of apoptosis, was specifically detected in cells expressing the VprS79A or VprK27M mutants (Fig. 7*B*). To analyze whether this cytotoxicity was the result of a defect in cell cycle progression, such as a block at the  $G_1$ /S transition or a DNA synthesis defect in S phase, we conducted a DNA synthesis assay at day 3 post-selection. We followed the incorporation of EdU, an analog of thymidine, into DNA. Cells expressing VprQ65R or wt Vpx exhibit a normal S phase, *i.e.* DNA content between 2N and 4N and efficient EdU incorporation (S, Fig. 7*C*). In contrast, cells expressing VprK27M or VprS79A present a “false” S phase, *i.e.* S phase-like DNA content but very poor EdU incorporation. The latter state has been referred to as “sub- $G_2$ ” and likely corresponds to 4N cells undergoing DNA fragmentation upon apoptosis (51). No significant  $G_1$  arrest could be detected

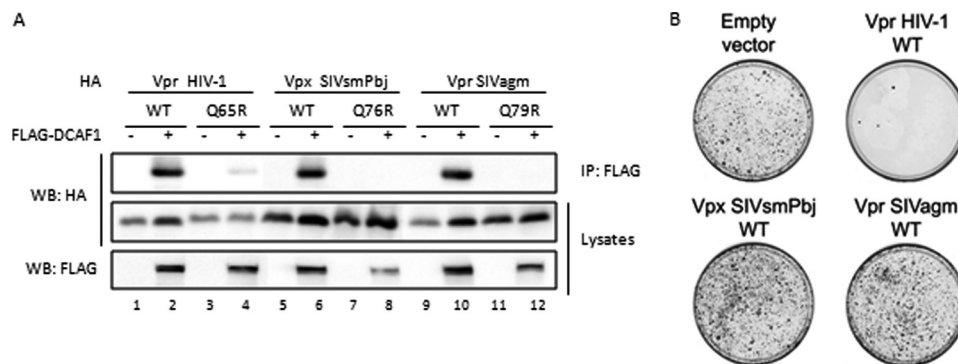


**FIGURE 4.  $G_2$  arrest-defective mutants of Vpr conserve cytotoxicity in a DCAF1 binding-dependent manner.** HeLa cells were transfected in duplicate wells of 12-well plates with EBV-based episomes expressing the indicated proteins. Two days after transfection, cells were harvested, and 1:10 of the cell population was transferred into 10-cm dishes and grown in hygromycin-containing medium. The formation of hygromycin-resistant colonies due to stable maintenance of the episome was visualized by Giemsa staining 15 days post-selection. The result of a representative experiment is shown in *A*, and quantification of six independent experiments is illustrated in the *bar graph* in *B*. The results of an additional experiment including three new double mutants are presented in *C*.

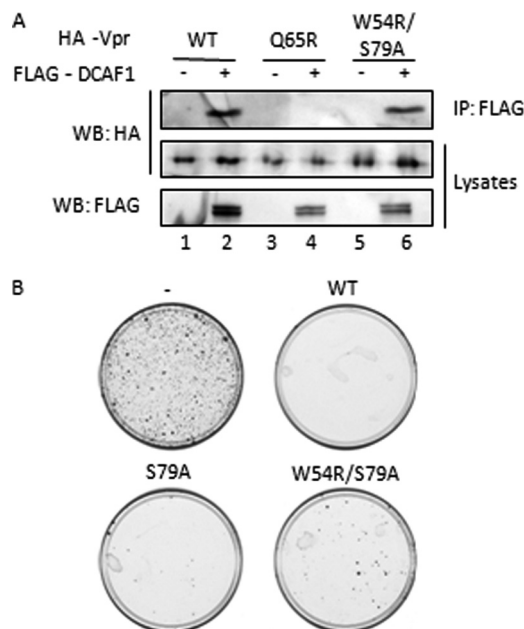
in these cells. Altogether, these data indicate that  $G_2$  arrest-defective but DCAF1 binding-proficient Vpr mutants trigger a cell death process, which differs from wt Vpr-induced cell killing by its slower kinetics and its lack of effect on cell cycle progression.

*$G_2$  Arrest-defective Vpr Mutants, Delivered through Virus-like Particles, Can Induce Apoptosis in Jurkat Cells*—Our data until now relied on experiments carried out in HeLa cells. We wondered whether the  $G_2$  arrest-independent cytopathicity of Vpr can also be observed in HIV target cells such as lymphocytes. We chose to deliver Vpr to Jurkat T cells through HIV-1 virus-like particles (VLP) to approach the physiological conditions of Vpr supply during infection. Vpr is actively encapsidated into virions through the association with the p6 gag prod-

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**FIGURE 5. The DCAF1 binding activity of the Vpr/Vpx superfamily is not sufficient to confer cytopathicity.** *A*, Vpx from SIVsmm and Vpr from SIVagm bind DCAF1 as efficiently as HIV-1 Vpr. The indicated constructs were transfected into HeLa cells. Cell lysates were prepared 48 h post-transfection and subjected to immunoprecipitation (IP) as described in Fig. 1*B*. *WB*, immunoblot. *B*, neither Vpx from SIVsmm nor Vpr from SIVagm can mediate cytopathicity. The colony formation assay was performed as described for Fig. 4.



**FIGURE 6. UNG2 does not mediate Vpr-induced cytopathicity in the clonogenic assay.** *A*, the UNG2 binding-deficient W54R/S79A Vpr mutant is still able to recruit DCAF1. The indicated constructs were transfected into HeLa cells. Immunoprecipitation (IP) was performed as described for Fig. 1*B*. *WB*, immunoblot. *B*, preventing binding between Vpr and UNG2 does not alter the cytopathic activity of Vpr. The colony formation assay was performed as described for Fig. 4.

uct and, thus, is delivered in cells upon virion entry (52–56). We first checked that Vpr and its mutants were well encapsidated into the VLP. The three  $G_2$  arrest-defective Vpr mutants, VprQ65R, VprK27M, and VprS79A, were incorporated into VLP as efficiently as wild-type Vpr (see Fig. 8*A*). Jurkat cells were incubated with VLP bearing Vpr or its mutants on a daily basis for 4 days. We measured the percentage of apoptotic cells through annexin-FITC staining at 48, 72, and 96 h after the first transduction (Figs. 8, *B* and *C*). As already reported (57, 58), wild-type Vpr from the incoming viral particle can induce apoptosis of Jurkat cells. Confirming the results obtained in HeLa cells, both VprK27M and VprS79A also exert an apoptotic activity in Jurkat cells but to a lesser extent than wild-type Vpr (25% Vpr K27M, 38% Vpr S79A, and 55% WT Vpr annexin-FITC-positive cells at the 96-h time point, respectively). Induction of apoptosis was not observed with VLP bearing VprQ65R

as well as with empty VLP. Therefore, Vpr displays a  $G_2$  arrest-independent but DCAF1 binding-dependent cytopathic activity in lymphocytes.

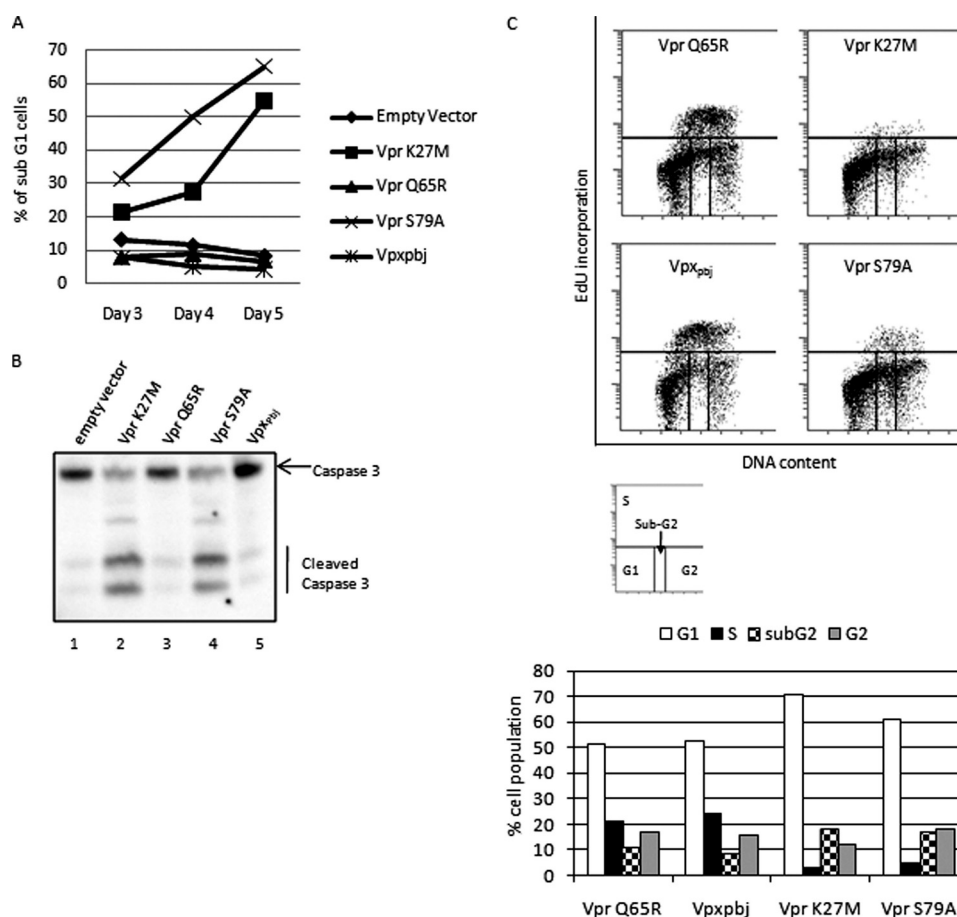
## DISCUSSION

The main findings of the present work are that (i) a highly conserved SRIG motif in Vpr is critical for Vpr-mediated  $G_2$  arrest and may be predictive of the ability of Vpr proteins from different origins to mediate  $G_2$  arrest, (ii) binding of Vpr to the  $G_2$  target requires nonlinear physical determinants, and (iii) the Vpr protein encoded by the HIV-1 virus uses two distinct pathways to impair cell growth, which both rely on DCAF1 binding.

The demonstration that the recruitment of the host DCAF1 ubiquitin ligase is critical for wt Vpr-mediated  $G_2$  arrest has allowed us to refine the genetic dissection of Vpr-associated cytopathic activities. We thus showed that the C-terminal tail of Vpr contains a highly conserved SRIG motif that is not involved in the recruitment of DCAF1 but is critical for the recruitment of the  $G_2$  target. Such results were previously described for the Ser-79 and Arg-80 residues in HIV-1 Vpr (20, 21) but not for the I and G residues. We hypothesized for the first time that the SRIG motif in the Vpr gene family may represent a signature of the  $G_2$  arrest activity. Supporting this hypothesis, we found that two Vpr proteins from simian viruses, SIVmnd-2 Vpr and SIVrcm Vpr, presenting a SRIG motif in their C-terminal tails, were able to promote cell cycle arrest in human cells, in contrast to SIVagm Vpr or SIVsmm Vpx (9–11). Moreover, the mutation of the serine residue in the SRIG motif abolished SIVmnd-2 Vpr-mediated  $G_2$  arrest (data not shown). We also obtained strong genetic evidence that the C-terminal tail of Vpr does not represent the unique determinant necessary for the recruitment of the  $G_2$  target; (i) mutations of Vpr falling into regions outside of the C-terminal tail abrogate  $G_2$  arrest activity without affecting DCAF1 recruitment and (ii) fusion of the Vpr C-terminal tail to the DCAF1 binding domain of SIVsmm Vpx or SIVagm Vpr did not restore wt Vpr-mediated  $G_2$  arrest. Thus, it appears that the core helical domain of Vpr also contributes to the recruitment of its  $G_2$  target.

The conclusion that HIV-1 Vpr uses two distinct pathways to impair cell growth is based on our demonstration that a series of  $G_2$  arrest-defective Vpr mutants conserve a cyto-





**FIGURE 7. G<sub>2</sub> arrest-independent cytotoxicity of Vpr correlates with induction of apoptosis but not with a block in cell cycle progression.** *A*, the expression of VprK27M or VprS79A induces an accumulation of cells in the sub-G<sub>1</sub> phase. HeLa cells were transfected with the indicated EBV constructs. 3, 4, and 5 days post-selection in hygromycin-containing medium, cells were harvested and analyzed for their cell cycle profiles as described in Fig. 1 legend. Flow cytometry analysis was performed with the total cell population to include cells with a DNA content <2N (sub-G<sub>1</sub>), indicative of an ongoing apoptotic death. The *bar graph* represents the proportion of cells in sub-G<sub>1</sub> over time. *B*, expression of VprK27M and VprS79A mediate activation of caspase 3 is shown. HeLa cells were transfected with the indicated EBV constructs. 4 days post-selection in hygromycin-containing medium, cells were harvested and lysed, and proteolytic activation of caspase 3 was analyzed by a Western blot. *C*, the long term cytopathic activity of Vpr is independent of a cell cycle blockage. HeLa cells were transfected with the indicated EBV constructs. After 3 days in hygromycin-containing medium, cells were incubated for 1 h with EdU, a BrdU analog that is incorporated into DNA during the S phase. Cells were then fixed, stained with propidium iodide, and analyzed by flow cytometry. The cell cycle status of the cells as presented at the *top* is explained by the *small graph in the middle*. The *bar graph at the bottom* represents for each Vpr mutant the percentage of cells in the different phases of the cell cycle.

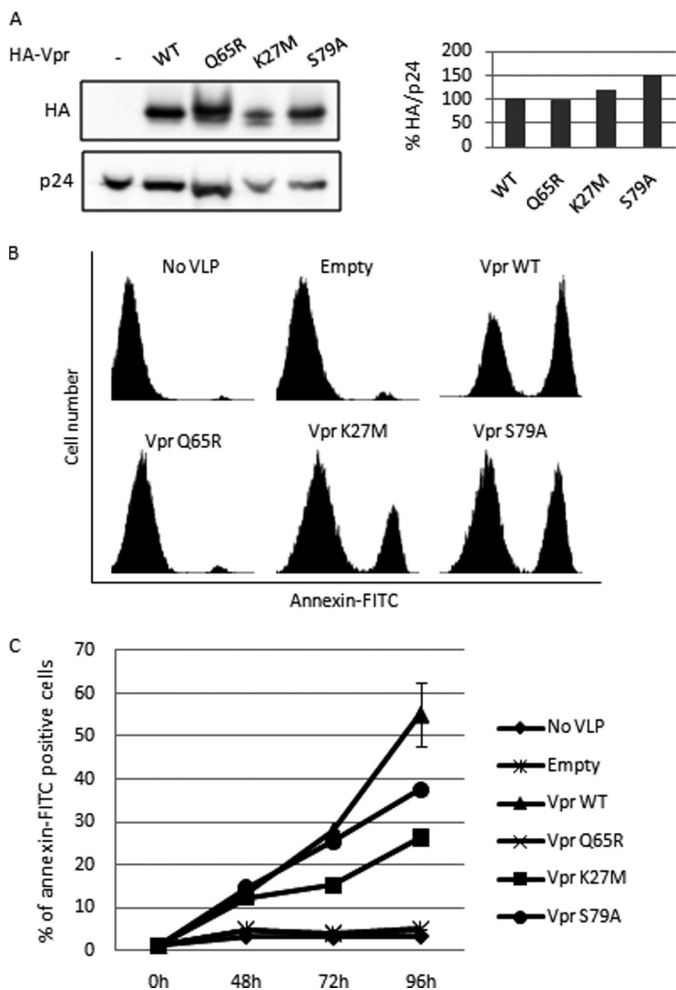
toxic activity. We further provide evidence that both pathways rely on DCAF1 binding to Vpr. Recruitment of DCAF1 by HIV-1 Vpr-related proteins, such as Vpr from SIV<sub>agm</sub> and Vpx from SIV<sub>smm</sub>, does not interfere with cell growth, ruling out that G<sub>2</sub> arrest-defective Vpr mutants maintain cytotoxic properties by inhibiting endogenous activity of DCAF1. These data support the hypothesis that Vpr inactivates two distinct cellular factors required for proper cell growth by recruiting DCAF1 (model presented in [supplemental Fig. 2](#)). A formal proof for this model would be that knocking-down the expression of DCAF1 would inhibit the long term cell death induced by Vpr, in the same manner that it inhibits Vpr-mediated G<sub>2</sub> arrest. Unfortunately, the strong toxicity we observed using DCAF1 siRNA for more than 4 days precluded such experiments (data not shown). This pitfall may be partially explained by the crucial role of DCAF1 in DNA replication (59). Thus, our demonstration that Vpr-mediated cytopathic effect involves DCAF1 relies on the behavior of the DCAF1 binding-defective Vpr Q65R mutant.

This mutant still binds UNG2 in a two-hybrid assay and is efficiently encapsidated into virions, which suggests that it has conserved some Vpr properties despite showing reduced dimerization efficiency and an absence of binding to chromatin (18).

So far, the nature of the cellular proteins targeted by Vpr remains a mystery. We wondered whether the expression of the cellular target involved in the G<sub>2</sub>-arrest independent cytotoxicity of Vpr might be induced by interferon, a characteristic of several antiviral factors (60–66). Our preliminary results suggest that it is not the case (data not shown). According to Belzile *et al.* (18), mutations of residues required for Vpr-mediated G<sub>2</sub> arrest, but not for its G<sub>2</sub> arrest-independent activity, abrogate Vpr binding to chromatin. These observations support the idea that the cytotoxicity target is not recruited on the chromatin, in contrast to, as shown by Belzile *et al.* (18), the G<sub>2</sub> arrest target.

That a viral protein induces the degradation of several host proteins is not unusual. The E6 protein from HPV usurps a

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**FIGURE 8. Vpr from the incoming virion induces apoptosis independently of its  $G_2$  arrest activity in Jurkat cells.** *A*, Vpr wt and mutants are similarly incorporated into HIV-1 pseudoparticles. Pseudoparticles were produced in the absence or presence of HA-tagged wt or mutant Vpr proteins. The presence of Vpr and the p24 capsid in the viral particle was analyzed by Western blot (left panel). The signals obtained in the left panel were quantified and expressed as HA/p24 ratios (right panel). *B* and *C*, VprK27M and VprS79A, but not VprQ65R, induce apoptosis in Jurkat cells. Jurkat cells were incubated with empty VLP or VLP containing the indicated HA-Vpr proteins each day until harvesting. Cells were harvested 48, 72, and 96 h after the first incubation, and the number of apoptotic cells was determined by flow cytometry after labeling with annexin-FITC. *Panel B* shows the distribution of cells versus the staining intensity at 96h. *Panel C* shows the percentage of annexin-FITC-positive cells versus time.

HECT host ubiquitin ligase to induce p53 degradation, which represents the first reported example of such a mechanism in the pathogen world (67). More recently, E6 has been shown to use the HECT ligase to promote the degradation of E6TP1 (68). In the case of Vif from HIV, the same Cullin 5 ubiquitin ligase is used to promote the degradation of APOBEC3G/F and a distinct host target important for cell growth (69–72). The recruitment of a host ubiquitin ligase by small viral proteins may provide an explanation for their pleiotropic effects because with only few binding sites for host targets, they can modulate distinct cellular pathways. Vpr may inactivate at least three proteins using the CRL4 complex; that is, the two cellular factors we suspect to be required for normal cell growth and UNG2 (24, 48, 49), which is neither required for  $G_2$  arrest (50) nor for long term cytotoxicity as shown here.

The  $G_2$  arrest-dependent and  $G_2$  arrest-independent cytotoxic effects of Vpr differ in their respective kinetics in HeLa cells, the latter requiring more than 5 days to take place in dividing cells as opposed to 3–4 days for the former. This may explain why only few groups have put forward a  $G_2$  arrest-independent cell death associated with Vpr expression (43, 45, 73, 74). Strikingly, the  $G_2$  arrest-independent cytotoxicity of Vpr we unravel here does not correlate with the accumulation of cells at the  $G_1$  phase of the cell cycle as reported by these groups using cellular systems different from ours (43, 44, 75). Several studies have also reported Vpr-mediated induction of the p21<sup>CIP1/WAF</sup> protein (76–78), which as a cyclin-dependent kinase inhibitor has been shown to inhibit either  $G_1/S$  or  $G_2/M$  transition (79). However, no clear correlation has been made between p21 and Vpr-mediated  $G_1$  or  $G_2$  arrest. Here, we could not detect p21 induction along with Vpr-mediated cytotoxicity (data not shown), suggesting that p21 is not involved in the  $G_2$  arrest-independent cytopathic effect of Vpr. The Vpr-induced cascade, which eventually leads to apoptotic cell death through caspase 3 activation, requires further investigation.

Vpr-mediated cytotoxicity was further observed in lymphocytes, which are natural targets of HIV. Nonetheless, the physiological significance of the cytotoxic activities of Vpr, regardless of their dependence on  $G_2$  arrest, remains puzzling. Neither HIV-1 nor HIV-2 shows a dependence on Vpr for infection of dividing cells. Sooty mangabeys, the natural hosts of SIVsm, do not show a depletion of CD4 T cells despite a high viremia (32). As mentioned above, the only cells where the absence of Vpr might impair HIV replication are macrophages, which are non-dividing cells (4–7). Therefore, the cytotoxic activities of Vpr revealed in dividing cells may be contingent to its genuine functions during infection. They should not, however, be regarded as epiphenomena as they proceed through genetically dissectible mechanisms. They teach us that the actual function of Vpr is most likely the destruction of host factors, which preclude optimal infection under certain circumstances. In this view, both the long known  $G_2$  arrest-dependent cytotoxicity of Vpr and the  $G_2$  arrest-independent pathway that we characterized here are valuable entry points for unraveling the still elusive functions of Vpr during HIV infection.

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