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Activation of the ATR Pathway by Human Immunodeficiency Virus Type 1 Vpr Involves Its Direct Binding to Chromatin In Vivo

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The human immunodeficiency virus type 1 (HIV-1) protein Vpr (viral protein R) arrests cells in the G_2 phase of the cell cycle, a process that requires activation of the ATR (ataxia-telangiectasia and Rad3-related) pathway. In this study we demonstrate that the expression of Vpr does not cause DNA double-strand breaks but rather induces ATR activation, as indicated by induction of Chk1 phosphorylation and the formation of γ-H2AX and 53BP1 nuclear foci. We define a C-terminal domain containing repeated H(F/S)RIG sequences required for Vpr-induced activation of ATR. Further investigation of the mechanism by which Vpr activates the ATR pathway reveals an increase in chromatin binding of replication protein A (RPA) upon Vpr expression. Immunostaining shows that RPA localizes to nuclear foci in Vpr-expressing cells. Furthermore, we demonstrate direct binding of Vpr to chromatin in vivo, whereas Vpr C-terminal domain mutants lose this chromatin-binding activity. These data support a mechanism whereby HIV-1 Vpr induces ATR activation by targeting the host cell DNA and probably interfering with normal DNA replication.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS, which is characterized by continual loss of $CD4^+$ T lymphocytes and enhanced susceptibility to opportunistic infections and malignancies. To achieve optimal replicative efficiency, HIV-1 manipulates host cell processes such as gene regulation, chromatin remodeling, signal transduction, and regulations of major histocompatibility complex class 1 surface expression, cell cycle, and apoptosis, as well as overcoming host antiviral mechanisms and targeting bystander cells (50, 54). These multiple activities of HIV are mediated by the specific interactions of viral proteins with various cellular components. As a complex retrovirus, HIV-1 encodes not only the essential structural proteins, Gag, Pol, and Env, but also several regulatory (Tat and Rev) and accessory (Vpr, Vif, Vpu, and Nef) proteins. These accessory proteins, while initially thought to be dispensable for infection, have now been shown to be important for HIV infectivity and pathogenesis in vivo (8, 16, 19, 20, 50). Among them, Vpr (viral protein R) (51) is unique in that it is incorporated in the HIV-1 virion at a high copy number (10), suggesting that it may play a significant role in the early stage of infection.

Vpr is a small (96-amino-acid) basic protein conserved in HIV-1, HIV-2, and simian immunodeficiency virus (55). Although the molecular mechanisms of Vpr function during viral replication remain elusive, it has some interesting biological activities. Vpr localizes to the nucleus of the infected cell and, together with other virion components, promotes nuclear transport of HIV-1 preintegration complex (7, 18, 25, 33, 39). This function is critical for HIV-1 replication in macrophages and other nondividing cells (4, 12, 25). Vpr can also modestly activate transcription of the HIV-1 long terminal repeat and other cellular promoters (2, 11, 56). Notably, Vpr has the capacity to arrest cell cycle at the G_2 phase (26, 37, 38, 44). Several studies have related this function of Vpr to HIV-1 replication and pathogenicity. For example, transcription from the viral long terminal repeat has been shown to be enhanced in G_2 regardless of whether the arrest was induced by Vpr or by other means (21), and the ability of Vpr to increase viral replication correlates with G_2 arrest (22). This suggests that the G_2 arrest induced by Vpr provides a favorable environment for virus production.

Accumulating evidence indicates that Vpr-induced G_2 arrest depends on signaling events analogous to the DNA damage response (24, 26, 41). Specifically, it requires activation of the ATR (ataxia-telangiectasia and Rad3-related)-mediated checkpoint signal pathway (45, 68). ATR, the kinase related to ATM (Ataxia-Telangiectasia-Mutated) and Rad3, belongs to a conserved family of phosphatidylinositol 3-kinase-like protein kinases. ATR plays an essential role in maintaining genome integrity. In response to a variety of DNA-damaging agents, ATR is activated and initiates signaling cascade by phosphorylating a broad range of downstream substrates, which in turn implement transcriptional regulation, checkpoint control, and DNA repair functions (1, 47, 53, 58, 67). In G_2/M checkpoint control, ATR-dependent activation of Chk1 kinase (23, 28, 64) leads to Cdc25A degradation (65) and Cdc25C cytosolic sequestration (36, 48). This prevents the dephosphorylation and activation of the cyclin-dependent kinase 1–cyclin B complex, resulting in arrest of cell cycle in G_2 phase.

Although previous studies have clearly demonstrated the utilization of the ATR pathway by Vpr, the molecular mechanism by which Vpr activates ATR is not known. It is not clear whether Vpr causes DNA lesions and thus indirectly activates the ATR pathway or whether Vpr directly binds ATR and/or

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its regulatory proteins, so altering the activity of ATR. Our studies reveal that Vpr binds to chromatin and suggest that Vpr interferes with ongoing DNA replication and in doing so activates the ATR-dependent replication checkpoint pathway following viral infection.

MATERIALS AND METHODS

Cell culture and genotoxic agents. HeLa and 293T cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. In experiments with drug treatment, cells were incubated in medium containing 2 mM hydroxyurea (HU) (Sigma) for 2 h prior to analysis. Ionizing radiation was performed using a ^{137}Cs source. UV light was delivered in a single pulse using a Spectrolinker system (Spectronics Corporation). If not indicated specifically otherwise, the cells were analyzed 1 h after exposure to ionizing radiation (IR) and UV.

Plasmids and transfection. pHR-Vpr (with an internal ribosomal entry site between Vpr and green fluorescent protein [GFP] as a marker) and pHR-GFP plasmids were described in previous reports (45). cDNA corresponding to full-length Vpr or C-terminally truncated Vpr (Vpr-ΔC; residues 1 to 67) was amplified using pHR-Vpr as a template and cloned into a modified pIRES2-enhanced GFP (EGFP) vector (Clontech) and pOZFHN (35) to generate Vpr expression vector with N-terminal FLAG tag. The Vpr mutants Vpr-H71A, Vpr-H71A/G75A, and Vpr-Δ (deletion of HFRIGC motif) were generated using a QuikChange site-directed mutagenesis kit (Stratagene). Transfection was performed using Lipofectamine 2000 (Invitrogen).

Viral vector transduction. Lentiviral vectors were produced and titrated as previously described (45, 68). To achieve greater than 90% efficiency, infections were performed at a multiplicity of infection of 2.5 with 10 μg/ml Polybrene (Sigma).

Antibodies. Rabbit polyclonal phospho-Chk1 (Ser317) and mouse monoclonal phospho-ATM (Ser1981) antibodies were purchased from Cell Signaling Technology, Inc. Rabbit polyclonal anti-γ-H2AX and anti-53BP1 antibodies were raised as described previously (40). Rabbit anti-HA antiserum was generated using hemagglutinin (HA) epitope peptide as an immunogen. Anti-FLAG M2 monoclonal antibody and anti-β-actin monoclonal antibody were purchased from Sigma. Mouse monoclonal antibody to RPA70 (Ab-1) was obtained from Oncogene. Rabbit polyclonal anti-Orc2 antiserum was purchased from PharMingen. Peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse immunoglobulin G and rhodamine Red-X- or fluorescein isothiocyanate-conjugated goat anti-mouse and goat anti-rabbit immunoglobulin G were from Jackson ImmunoResearch.

Western blotting. Cells were harvested and lysed in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% Nonidet P-40), and the insoluble fraction was pelleted for 10 min in a microcentrifuge. Protein samples were then subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membrane was blocked with 5% nonfat milk for 1 h prior to incubation with primary antibodies for 2 h at room temperature or overnight at 4°C. The blots were washed in Tris-buffered saline containing 0.2% Tween 20, incubated with peroxidase-conjugated secondary antibodies, and visualized by chemiluminescence using a SuperSignal kit (Pierce).

Cell cycle analysis. Cells transfected with wild-type or mutant Vpr in pIRES2-EGFP vector (Clontech) or with EGFP alone were harvested at 36 h posttransfection. The cells were washed in phosphate-buffered saline (PBS) and fixed in 0.3% paraformaldehyde on ice for 30 min, following permeabilization with 0.2% Triton X-100 for several hours. After being washed with PBS twice, the cells were treated with RNase A (500 U/ml) for 1 h at 37°C and stained with propidium iodide (25 μg/ml) for 30 min at 37°C. Cell cycle profiles of GFP-positive cells were analyzed by flow cytometry with CellQwest and Modifit software.

Immunofluorescence microscopy and in situ detergent extraction. Cells grown on glass coverslips were fixed with 3% paraformaldehyde for 10 min at room temperature followed by incubation with 0.5% Triton X-100 for 5 min. Immunostaining was performed with the combinations of primary and secondary antibodies (diluted in 5% goat serum) for 20 min each at 37°C as specified in the figure legends. For visualization of replication protein A (RPA) accumulation on chromatin, cells were permeabilized with 0.5% Triton X-100 for 5 min prior to fixation. For Vpr nuclear retention experiments, transfected cells were subjected to 0.5% Triton X-100 extraction for 5 min followed by 3% paraformaldehyde fixation or were fixed in a 1:1 methanol-acetone solution for 5 min. In the indicated group, cells were incubated with micrococcal nuclease (50 units/ml) in PBS plus calcium and magnesium for 10 min at 37°C prior to fixation. Cells were counterstained for nuclear DNA with 0.1 μg/ml DAPI (4′,6′-diamidino-2-phenylindole), mounted, and viewed with a Nikon ECLIPSE E800 fluorescence microscope using a 40× or 60× objective. Images were processed using Adobe Photoshop and Illustrator software.

Chromatin fractionation. Chromatin fractionations were performed as described previously (69), with modifications. Briefly, 3×10^6 cells were collected, washed with PBS, and resuspended in 200 μl of solution A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol, 10 mM NaF, 1 mM Na_2VO_3, protease inhibitors) with 0.5% Triton X-100. Cells were incubated on ice for 5 min followed by low-speed centrifugation (1,300 × *g*, 4 min) to separate cytoplasmic proteins from nuclei (P1). Isolated nuclei were then washed twice with solution A followed by resuspension in solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, protease inhibitors) to extract soluble nuclear proteins. After incubation on ice for 10 min, soluble nuclear proteins were separated from chromatin (P2) by centrifugation (1,700 × *g*, 4 min). After two washes with solution B, isolated chromatin was spun down by high-speed centrifugation (10,000 × *g*, 1 min). Finally, chromatin was resuspended in 100 μl of sodium dodecyl sulfate sample buffer and sheared by sonication on ice for 15 s to extract chromatin-bound proteins. For micrococcal nuclease digestion, nuclei (P1) were resuspended in solution A containing 1 mM $CaCl_2$ and 50 U of micrococcal nuclease. After incubation at 37°C for 2 min, digested nuclei were fractionated as stated above.

Pulsed-field gel electrophoresis (PFGE) assay. For determination of double-strand breaks (DSBs), equal numbers of cells were embedded in agarose plugs and lysed for 16 h at 50°C in 1% sarcosyl (*N*-lauroyl-sarcosine; Sigma)–0.5 M EDTA–1 mg/ml proteinase K (Invitrogen). The plugs were washed in Tris-EDTA buffer, and electrophoresis was performed with a CHEF DRII system (Bio-Rad Laboratories) for 65 h in 0.8% agarose in 0.5× Tris-borate-EDTA at 14°C with a field strength of 1.5 V/cm and pulse times increasing from 50 to 5,000 s. The gel was then stained with 1 μg/ml ethidium bromide for 20 to 30 min, washed with water, and imaged. The level of DNA breakage was estimated by the fraction of DNA migrating from the plug into the gel.

RESULTS

Vpr induces ATR activation. Previous results suggest that Vpr-induced G_2 arrest requires the ATR-mediated signaling pathway. Thus, it is likely that ATR is activated following Vpr expression. To confirm this, we analyzed phosphorylation or activation of several ATR substrates. A key substrate of ATR involved in G_2/M checkpoint signaling is the Chk1 protein kinase, which is phosphorylated by ATR on Ser317 and Ser345 (27, 64) in response to DNA damage or replication blocks induced by agents such as HU. The induction of Chk1 phosphorylation on Ser345 by Vpr has previously been shown (45). In addition to this, we also examined phosphorylation of Chk1 on Ser317. 293T cells were infected with lentivirus vectors expressing either Vpr with GFP as a marker or control GFP. In similarity to the results seen with cells treated with HU, Vpr-infected cells exhibited an increased level of Ser317-phosphorylated Chk1 compared with mock-treated or GFP-infected cells (Fig. 1A), suggesting that Chk1 is activated following Vpr expression.

ATR-mediated phosphorylation of Chk1 is dependent on the "9-1-1" (Rad9-Rad1-Hus1) complex (62, 69), which might act to enable ATR to recognize its substrates on the chromatin. It was not clear whether the observed stimulation of Chk1 phosphorylation by Vpr represents an effect on the regulation of ATR substrate selection through the "9-1-1" complex or whether Vpr acts upstream of ATR. To clarify this point, we examined another ATR-dependent phosphorylation event that is independent of the "9-1-1" complex. H2AX is a histone protein variant that represents 2 to 25% of histone H2A and is randomly incorporated in nucleosomes (17). In response to DNA damage, it undergoes immediate phosphorylation and forms nuclear foci (referred to as γ-H2AX foci) (17, 42, 43).

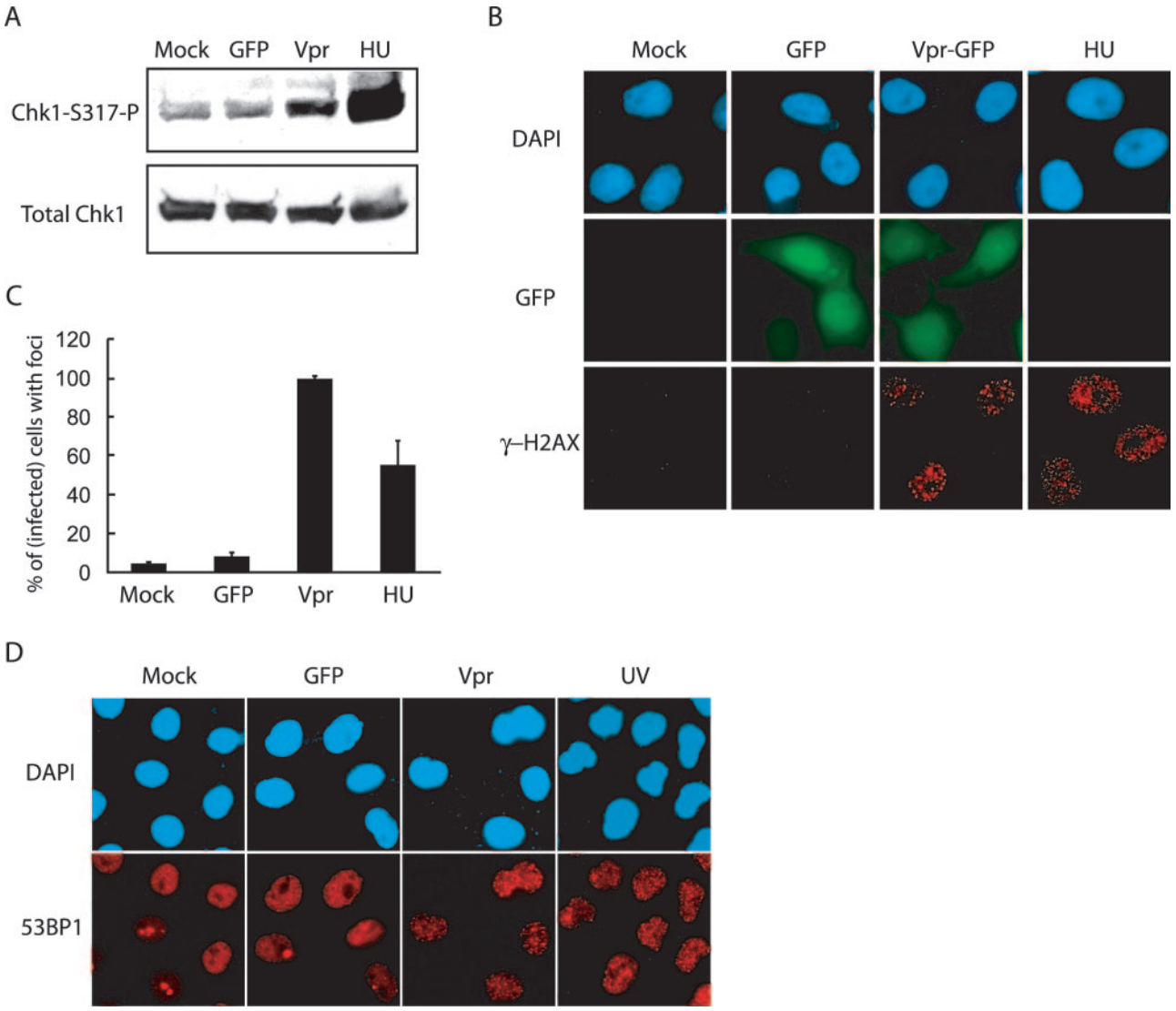

FIG. 1. Vpr activates the ATR-dependent pathway in vivo. 293T cells (A) or HeLa cells (B to D) were mock infected, infected with lentivirus vectors encoding Vpr with GFP as a marker (Vpr-GFP) or with GFP alone, or treated with 2 mM HU or UV (40 J/m^2). At 36 h after transduction, cells were lysed and analyzed by immunoblotting with the phosphospecific antibody to P-Ser317 of Chk1 (A) and fixed and immunostained with phospho-H2AX (γ-H2AX) antibody and 53BP1 antibody (B and D). (C) Quantification of percentages (means ± standard deviations from three replicate infections) of infected, GFP-positive cells with γ-H2AX foci is summarized.

This process has been reported to be ATR dependent following replication stress. Moreover, H2AX phosphorylation by ATR is distinct from Chk1 phosphorylation since it is independent of Hus1, a member of the "9-1-1" complex (59). In similarity to the results seen with HU-treated cells, we found that cells infected with Vpr vector (as indicated by the GFP marker) exhibited an increased γ-H2AX immunostaining pattern (Fig. 1B). By quantifying cells with intense γ-H2AX foci, we observed that more than 95% of Vpr-expressing cells were γ-H2AX focus positive, whereas less than 8% of cells in the mock- or GFP-infected group were found positive for γ-H2AX foci (Fig. 1C). As a control, ~50% of HU-treated cells have positive γ-H2AX foci, representing the S population in these cells.

γ-H2AX staining appears to be very sensitive assay for analyzing the Vpr-induced ATR activation. To further confirm our finding, we examined 53BP1 localization, another marker for DNA damage and/or replication stress. In response to replication stress, 53BP1 rapidly redistributes to discrete foci that colocalize with γ-H2AX. This redistribution of 53BP1 is dependent on ATR (59, 60). We observed similar induction of 53BP1 nuclear foci in Vpr-infected and UV-irradiated cells (Fig. 1D). Collectively, these results suggest that in similarity to DNA replication stress, Vpr expression leads to ATR activation.

The C-terminal domain of Vpr is required for Vpr-induced activation of the ATR pathway. Previous studies of *Saccharomyces cerevisiae* and mammalian cells by use of various Vpr mutants defined the C-terminal region of Vpr responsible for its G_2 arrest function (6, 30, 31, 34). Specifically, a highly

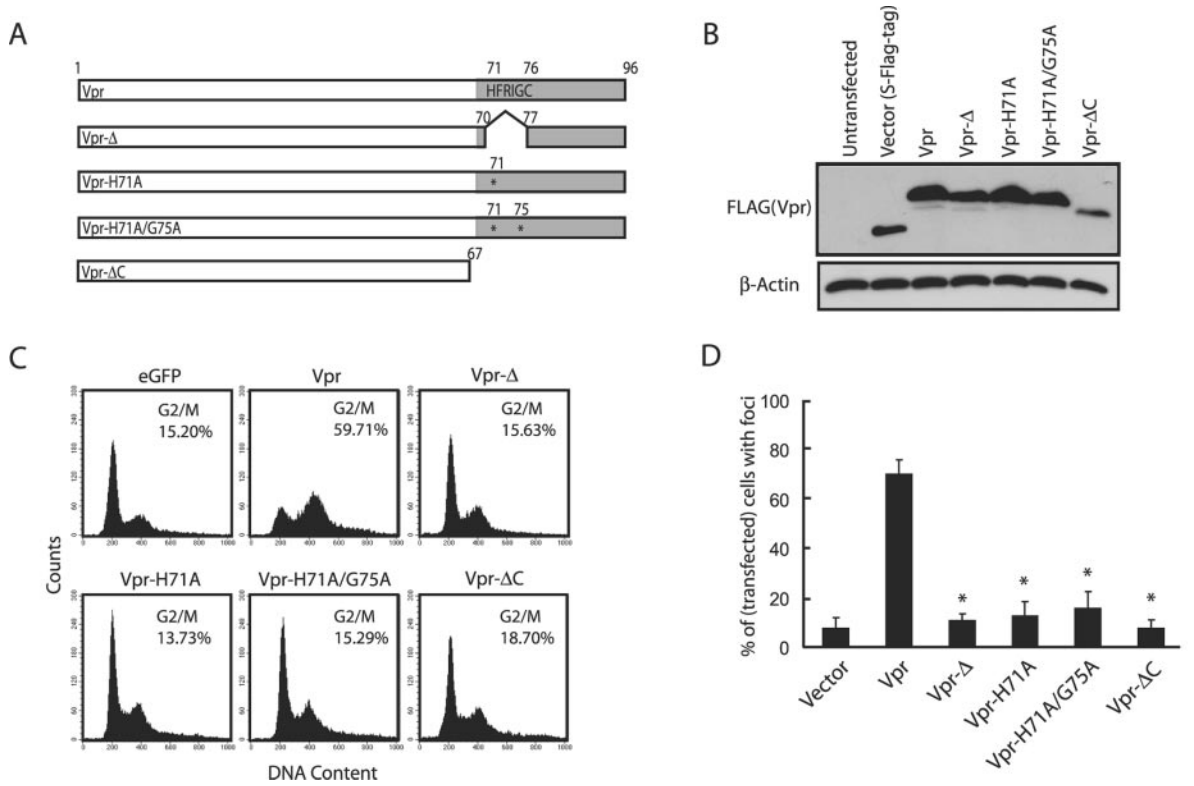

FIG. 2. A C-terminal domain containing H(S/F)RIG motifs is required for Vpr-induced ATR activation. (A) Schematic representation of the wild-type and mutant Vpr constructs that were N-terminally fused to a FLAG epitope tag (* denotes point mutations). (B to D) HeLa cells were transfected with pIRES2-EGFP vector expressing various forms of FLAG-tagged Vpr as shown in panel A. (B) Vpr expression was analyzed by immunoblotting with anti-FLAG antibody. The vector encoding S-FLAG tag only was included, and β-actin was blotted as a loading control. (C) The cell cycle profiles of vector (EGFP), wild-type Vpr, or mutant Vpr-transfected cells were analyzed 36 h after transfection. Consistent data were obtained in three independent experiments. (D) Cells were costained with γ-H2AX and anti-FLAG antibodies, and the percentages (means ± standard deviations from four replicates) of Vpr-transfected cells with γ-H2AX foci were quantified (*, $P < 0.05$).

conserved HFRIGC motif was reported to be essential for this activity. Since Vpr-induced G_2 arrest is dependent on the activation of ATR, we reasoned that this same region of Vpr may also be critical for ATR activation. To test this hypothesis, we generated FLAG-tagged Vpr lacking the HFRIGC motif (Vpr-Δ), with a single-point mutation on residue His71 (Vpr-H71A), with double-point mutations on residues His71 and Gly75 (Vpr-H71A/G75A), or with C-terminal truncation (Vpr-ΔC) (Fig. 2A and B). Although these mutants have been shown to be defective for Vpr-mediated growth arrest in yeast systems, their importance in mammalian cells, particularly the requirement of residues His71 and Gly75, remains in question (15, 22, 31). We therefore transfected HeLa cells with these mutants and compared their G_2 arrest activities with that of the wild-type Vpr. The dramatic shifting of cell cycle profile toward G_2 phase was only observed in the cells expressing wild-type Vpr but not in any of the Vpr mutants (Fig. 2C), confirming that the C-terminal domain and the residues we examined are critical for Vpr's G_2 arrest activity. We further analyzed γ-H2AX focus formation to evaluate the importance of this region in activating ATR. In contrast to wild-type Vpr, transfection with these Vpr mutants demonstrated significant reduction in populations with γ-H2AX foci (Fig. 2D). These results indicate that this region of Vpr is required for the induction of ATR activation.

Vpr expression does not cause DNA DSBs. We are interested in how Vpr activates the ATR pathway. One possibility is that the presence of Vpr in the nucleus might directly or indirectly lead to modifications of DNA or chromatin structure, which are in turn recognized by damage-sensor proteins or ATR itself, and so elicit the checkpoint signaling. The first issue we addressed is whether or not Vpr would directly or indirectly lead to the generation of DNA DSBs. We performed PFGE assays to examine DSB formation following Vpr expression. As shown in Fig. 3A, we detected significant numbers of DSBs in cells exposed to IR. In contrast, no lower-molecular-weight, fragmented DNA was observed in cells transfected with pHR-VPR (where transfection efficiency was >70%) or in control cells or in cells transfected with control pHR-GFP plasmid. These results suggest that Vpr expression does not induce a significant amount of DSBs in the cell.

ATR responds to DSBs and UV-induced lesions and also stalled replication forks, whereas ATM preferentially responds to DSBs (1, 52, 53). Therefore, to further inspect whether there was generation of DSBs associated with Vpr expression, we examined ATM activation by assaying the autophosphorylation at the Ser1981 site (3). Unlike IR-treated cells, which showed a dramatic increase in phosphorylated ATM staining, neither Vpr-transfected cells nor control cells showed significant activation of ATM (Fig. 3B). These studies imply that

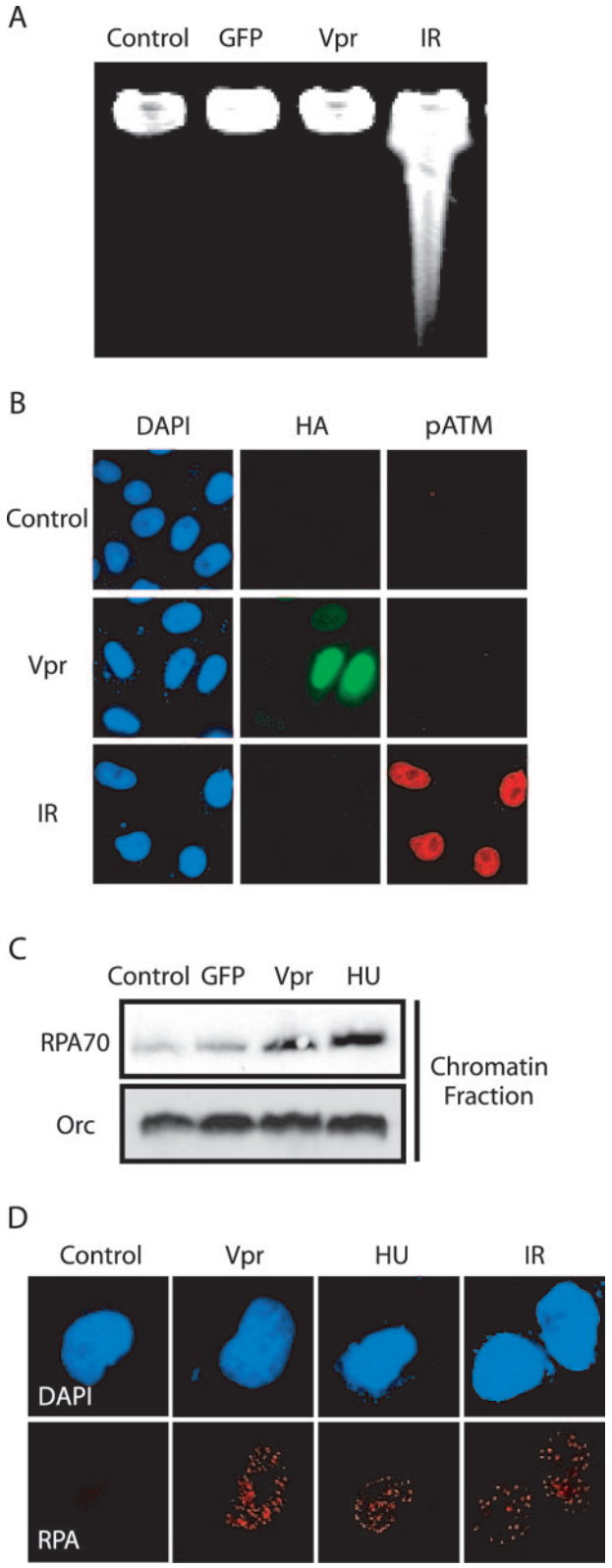

FIG. 3. Vpr does not cause DNA double-strand breaks but promotes RPA chromatin loading. (A) HeLa cells transfected with GFP or Vpr, or treated with 10 Gy of IR, were embedded in agarose plugs and lysed and then subjected to PFGE analysis for the examination of DNA double-strand breaks. (B) HeLa cells were mock treated, transfected with HA-tagged Vpr, or treated with 10 Gy of IR. Cells were fixed and costained with anti-HA and anti-P-Ser1981-ATM antibodies. (C) HeLa cells were transfected with GFP or Vpr, or treated with 2 mM HU, and then subjected to chromatin fractionation. Chromatin-associated RPA70 and Orc2 (which served as a loading control) were detected by immunoblotting. (D) HeLa cells were transfected with Vpr or treated with 2 mM HU or 10 Gy of IR. Cells were briefly extracted with detergent, fixed, and immunostained with anti-RPA70 antibody.

DSBs are probably not responsible for Vpr-induced ATR activation. This is consistent with the previous finding that ATM is dispensable for Vpr-induced G_2 arrest (5, 68).

Vpr promotes RPA chromatin association. To determine whether Vpr might activate ATR via the damage sensor-ATR signaling scheme or through an alternative signaling pathway, we decided to analyze the effects of Vpr expression on some of the known upstream events required for ATR activation. Increased amounts of chromatin-associated RPA were observed after DNA damage and/or replication stress (29, 32, 70). Recent studies have demonstrated that the presence of the RPA-coated single-stranded DNA (ssDNA) is a preceding event required for the recruitment and activation of ATR–ATR-interacting protein complex in vitro and in vivo (13, 70). We therefore examined whether Vpr expression would result in an enhancement of RPA chromatin loading. We isolated chromatin fractions from untreated, GFP- or VPR-transfected, or HU-treated HeLa cells. An increased amount of chromatin-associated RPA was detected in Vpr-expressing cells similar to that observed in HU-treated cells (Fig. 3C). Such promotion of RPA chromatin association can also be visualized by using immunofluorescence. RPA localizes to nuclear foci after HU or IR treatment (Fig. 3D). These represent the formation of accumulated RPA-coated ssDNA structure at the stalled replication forks or processed DSBs. Cells with Vpr expression also displayed increased RPA foci (Fig. 3D), indicating that Vpr stimulates the recruitment of RPA to chromatin.

Vpr directly binds to chromatin in vivo. The next issue is how Vpr promotes RPA loading. Previous in vitro studies suggested that Vpr may have nucleic acid binding activity (9, 14, 63). Thus, we investigated whether Vpr could associate with chromatin in vivo and whether such an association correlates with its effects on ATR activation. Following in situ extraction of cells transfected with FLAG-tagged Vpr, a subpool of Vpr was found to be retained in the nucleus (Fig. 4A). This nuclear retention of Vpr can be observed as early as 6 h after transfection (data not shown), suggesting that Vpr may directly bind to chromatin. We further demonstrated that Vpr associates with chromatin but not with other nuclear matrix. We fractionated extracts of Vpr-transfected cells into fractions of cytoplasmic proteins, soluble nuclear proteins, and chromatin-associated proteins. As shown in Fig. 4B, a portion of Vpr was detected in the chromatin fraction. Digestion of chromosomal DNA with micrococcal nuclease depleted Vpr from this chromatin fraction. This was accompanied by an increase of Vpr in the soluble nuclear fraction, suggesting that Vpr is indeed associated with chromatin. Interestingly, the Vpr C-terminal mutants, which are defective for ATR activation and G_2 arrest (Fig. 2), still localize to the nucleus but are dramatically di-

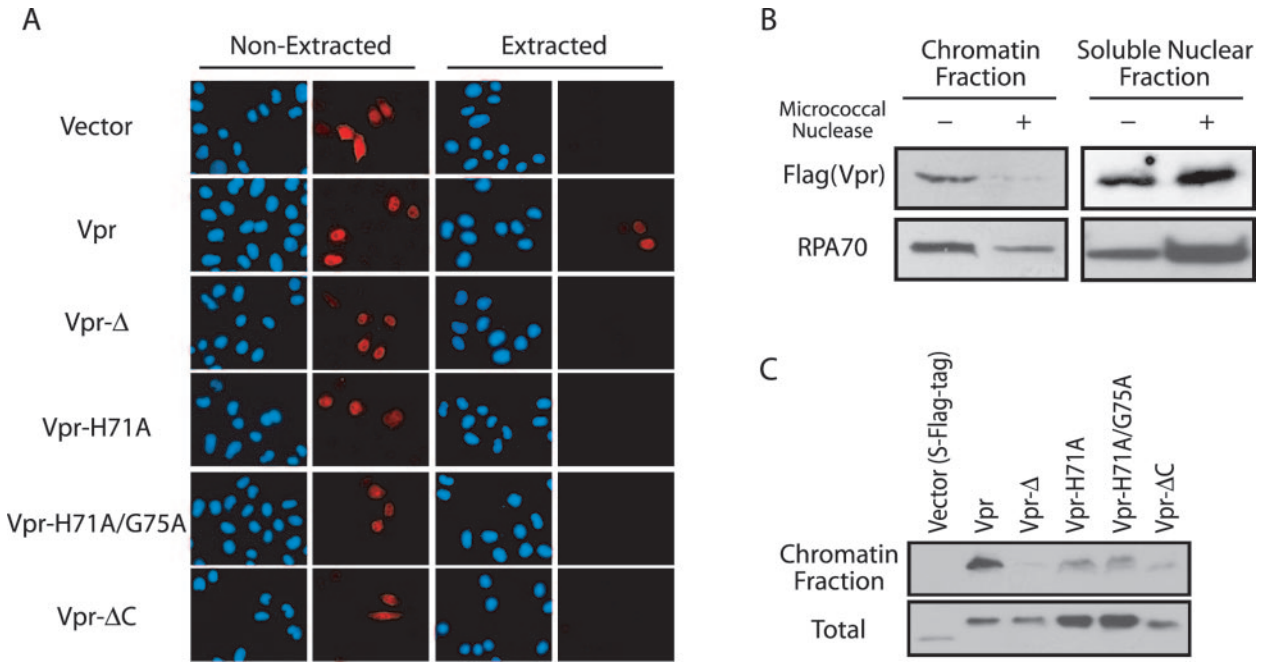

FIG. 4. Vpr binds to chromatin in vivo. (A) HeLa cells were mock transfected or transfected with FLAG-tagged wild-type or mutant Vpr. Nuclear retentions were assayed by in situ detergent extraction prior to immunostaining with FLAG antibody (red). (B to C) HeLa cells transfected with Vpr were subjected to chromatin fractionation. (B) Vpr and RPA70 in the indicated fractions with or without micrococcal nuclease treatment were detected by immunoblotting with anti-FLAG and anti-RPA antibodies. (C) Comparison of wild-type and mutant Vpr in chromatin association. The protein samples of 10% isolated chromatin fraction and 5% whole-cell lysate were immunoblotted with anti-FLAG antibody.

minished in extraction-resistant nuclear staining (Fig. 4A) or chromatin association (Fig. 4C).

To further examine the chromatin binding activity of Vpr, we used another Vpr expression vector (pOZFHN-Vpr) that has reduced Vpr expression and repeated the above-described in situ nuclear extraction experiments. Interestingly, the majority of cells with Vpr retention exhibited discrete nuclear foci localization of Vpr (Fig. 5A). Again, those foci were sensitive to nuclease treatment (Fig. 5A), demonstrating the binding and potential accumulation of Vpr on certain regions of chromatin. Moreover, a significant portion of those Vpr foci colocalizes with γ-H2AX foci (Fig. 5B), suggesting that Vpr associates with chromatin and thus indirectly activates the ATR-H2AX pathway.

DISCUSSION

In this study, we focused on the HIV-1 gene product Vpr and its interference with host cell function, specifically, the mechanism by which it activates the ATR-mediated DNA damage-signaling pathway. Our results provide evidence linking ATR activation to Vpr expression. In similarity to the results seen with DNA damage response, this process also involves the accumulation of chromatin-associated RPA. This led us to explore whether the HIV-1 Vpr actually causes damages on the host cellular DNA. Although no double-stranded DNA breaks were detected following Vpr expression, we observed the direct binding of Vpr to DNA or chromatin at distinct foci, some of which colocalize with γ-H2AX foci. The mutational analysis of Vpr further suggests the potential link between this DNA-chromatin binding activity of Vpr and its ability to activate ATR. On the basis of these observations, we propose the following model for Vpr-induced ATR activation: upon expression of Vpr and localization of Vpr to the host nucleus, a subpool of Vpr binds to DNA or chromatin, probably interfering with DNA replication. This in turn leads to the formation of RPA-coated ssDNA structures and activation of the ATR-dependent replication checkpoint pathway and results in cell cycle arrest in G_2 phase.

It is still not clear how Vpr facilitates the chromatin accumulation of RPA. Increased RPA chromatin binding occurs following DNA double-strand breaks or replication stress. So far, there is no evidence supporting the hypothesis that Vpr expression leads to the generation of DNA double-strand breaks. γ-H2AX foci visualized by immunofluorescence staining could be markers of megabase domains containing DNA DSBs (17, 42, 43). It is estimated that 1 Gy of IR produces about 35 DSBs per cell (46, 49). On average, we observed more than 50 γ-H2AX foci in Vpr-expressing cells. However, by a PFGE assay, we did not see levels of DNA breaks in Vpr-expressing cells comparable to the levels in cells treated with 2 Gy of IR. Additionally, if Vpr causes DSBs, one would expect to observe ATM activation. However, we did not detect any augmentation in ATM autophosphorylation in Vpr-expressing cells, which agrees with earlier studies suggesting that ATM is dispensable for Vpr-induced G_2 arrest (5, 68).

The most likely hypothesis is that Vpr induces replication stress and thus activates the ATR-mediated G_2 checkpoint pathway, as suggested by the increased chromatin-associated RPA levels following Vpr expression. We further demonstrated that Vpr localizes to nuclear foci and partially colocal-

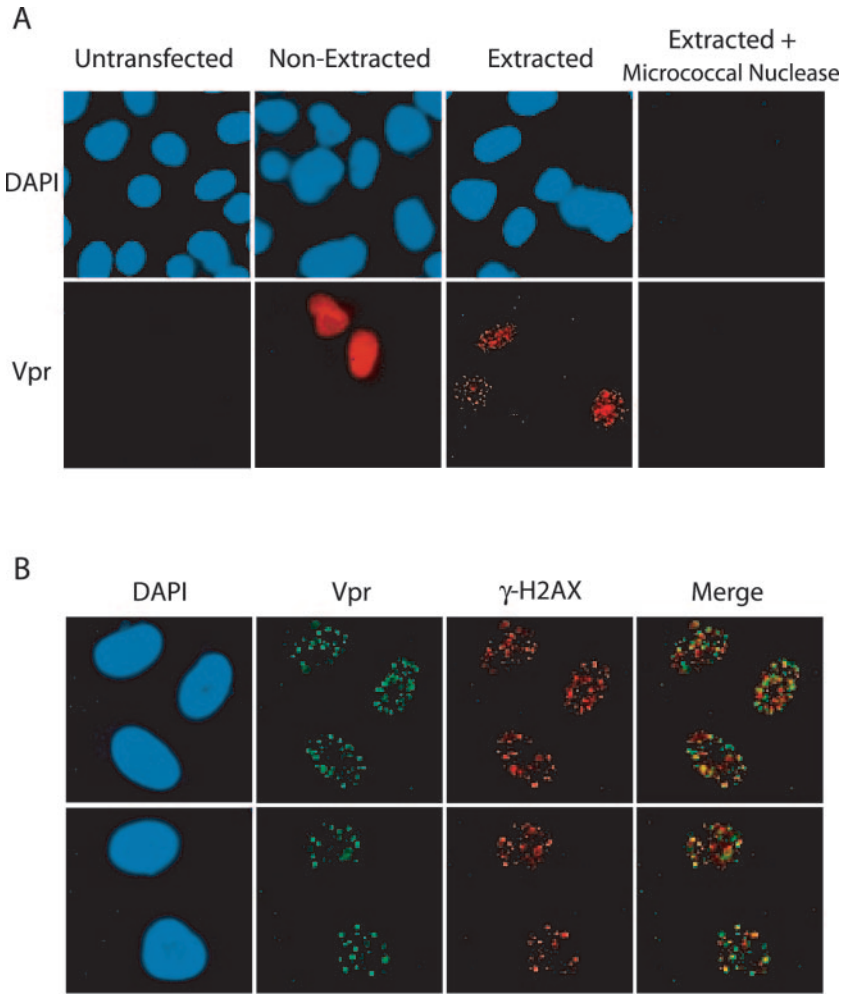

FIG. 5. Formation of extraction-resistant nuclear foci of Vpr. HeLa cells transfected with pOZFHN-Vpr (with a FLAG tag) were extracted for the analysis of chromatin-associated Vpr. (A) Cells with or without micrococcal nuclease treatment were extracted and stained with anti-FLAG antibody to visualize the accumulation of Vpr at distinct regions in nuclei. (B) Colocalization of Vpr foci with γ-H2AX foci was determined by immunostaining with indicated antibodies.

izes with γ-H2AX, implying that Vpr may directly bind to DNA and thus interfere with normal cellular DNA replication. Vpr has roles in promoting proviral DNA nuclear transport and transcriptional activation, and previous studies revealed that Vpr has intrinsic DNA binding activity in vitro (14, 63). Structurally, Vpr contains an N-terminal domain which may be involved in oligomerization (66), a leucine-zipper-like domain spanning residues 61 to 81 (57), and a flexible C-terminal domain (61). The C-terminal domain has abundant basic residues and is proposed to form an alpha-helix structure that may mediate the interaction of Vpr with DNA (9, 61). Here, we have shown that Vpr binds to chromatin in vivo and that this binding is dependent on the C-terminal domain. The chromatin-binding activity of Vpr correlates with its in vitro DNA binding activity and also with its ability to activate the ATR pathway and induce G_2 cell cycle arrest. These observations suggest that Vpr may bind at distinct sites throughout chromatin and thus interfere with normal DNA replication. It remains to be determined whether Vpr specifically recognizes certain DNA sequences or whether it prefers certain DNA structures.

Current therapy for HIV-1 infection relies largely on the inhibition of HIV-1 proteases and integrase. Because the HIV-1 regulatory and accessory proteins play important roles at various stages of the viral life cycle, these viral proteins may be used as new targets for future antiviral therapies. Indeed, Vpr-induced G_2 arrest is critical for efficient HIV-1 replication and cytopathicity. It is conceivable that pharmacological prevention of Vpr-induced G_2 arrest may yield new approaches for therapeutic intervention. Of course, the ATR pathway is the obvious target for drug design. However, since the ATR-Chk1 pathway is also critical for the maintenance of genome stability, blocking this pathway may generate unwanted consequences (for example, promoting neoplastic transformation). Here, we demonstrate that Vpr binds DNA or chromatin in vivo and that this DNA- or chromatin-binding activity of Vpr is linked with its ability to activate the ATR pathway. Thus, the DNA binding activity of Vpr provides a potential specific viral target that is unlikely to result in any cellular toxicity or side effect. To achieve this goal, we will first need to understand how Vpr interacts with DNA at the molecular level.

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