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Activation of the ATR-mediated DNA Damage Response by the HIV-1 Viral Protein R*

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DNA damage is a universal inducer of cell cycle arrest at the G_2 phase. Infection by the human immunodeficiency virus type 1 (HIV-1) also blocks cellular proliferation at the G_2 phase. The HIV-1 accessory gene *vpr* encodes a conserved 96-amino acid protein (Vpr) that is necessary and sufficient for the HIV-1-induced block of cellular proliferation. In the present study, we examined a recently identified DNA damage-signaling protein, the ATM- and Rad3-related protein, ATR, for its potential role in the induction of G_2 arrest by Vpr. We show that inhibition of ATR by pharmacological inhibitors, by expression of the dominant-negative form of ATR, or by RNA interference inhibits Vpr-induced cell cycle arrest. As with DNA damage, activation of ATR by Vpr results in phosphorylation of Chk1. This study provides conclusive evidence of activation of the ATR-initiated DNA damage-signaling pathway by a viral gene product. These observations are important toward understanding how HIV infection promotes cell cycle disruption, cell death, and ultimately, CD4+ lymphocyte depletion.

DNA damage-signaling pathways consist of a network of interacting and occasionally redundant signals that may lead to the inactivation of the Cdc2-cyclin B complex (1–5) and cell cycle arrest in G_2. A major point of regulation of the Cdc2-cyclin B cyclin complex is through inhibitory phosphorylation of Cdc2 on Tyr-15. Phosphorylation of the adjacent residue, Thr-14, also contributes to the inhibition of Cdc2 activity. Cdc25C is a dual specificity phosphatase that dephosphorylates Cdc2 on both Tyr-15 and Thr-14, leading to Cdc2 activation. Upon induction of the DNA damage checkpoint Cdc25C is inactivated through the actions of several kinases, including Chk1 and Chk2, which are under the control of the phosphatidylinositol 3-kinase-like proteins ATR and ATM.

ATR and ATM respond to a variety of abnormal DNA structures and initiate a signaling cascade leading to a DNA damage checkpoint (6). Their roles are partially redundant but with some important distinctions both with regard to substrate preference and the types of the DNA damage to which the kinases respond. In response to genotoxic stress, ATM is responsible for phosphorylation of the Chk2 protein kinase, whereas ATR phosphorylates Chk1. ATR is primarily responsible for enforcement of the cell cycle checkpoint activated in response to intra-S-phase genotoxic stress, as exemplified by stalled replication forks and topoisomerase inhibition (7, 8). In contrast, ATM is more important for the ionizing radiation-induced DNA damage checkpoint. Both proteins are inhibited by methylxanthines, such as caffeine. ATR acts in concert with Rad17 and the proliferating cell nuclear antigen-like heterotrimer composed of Rad9, Hus1, and Rad1 to enforce the DNA damage checkpoint (9–11).

ATR deletion is lethal early in embryogenesis (12, 13). Therefore, in mammalian cells, ATR function must be studied by the introduction of an ATR kinase inactive mutant (14–16) in an inducible manner or by transient knockdown of ATR expression via RNA interference (RNAi).

Several human viruses including reovirus (17), human *Papillomavirus* (18), and the human and simian immunodeficiency viruses (19) encode genes that activate the G_2 checkpoint. The induction of cell cycle arrest by the HIV-1[1] *vpr*-related genes of primate lentiviruses have been most extensively studied (20–25). Vpr-induced G_2 arrest leads to moderate transactivation of the HIV-1 promoter, the long terminal repeat (LTR) (26–29). The G_2 phase arrest and subsequent apoptosis may explain aspects of the CD4+ cell death in HIV infection.

Early studies demonstrated that Vpr-induced G_2 arrest is associated with inactivation of the cyclin-dependent kinase, Cdc2, by hyperphosphorylation and concomitant suppression of Cdc2-cyclin B kinase activity that is necessary for the G_2 to M transition (21–23). In response to Vpr, the Cdc2-specific phosphatase, Cdc25C, is hyperphosphorylated in a pattern consistent with inactivation (21). Induction of G_2 arrest by Vpr can be overcome by methylxanthines (27, 30). Together, the above observations have led to the suggestion that Vpr induces cell cycle arrest via a DNA damage-sensitive pathway (30), although the precise signaling pathway has remained elusive. A direct binding of Vpr to DNA has been reported (31). However the possibility that Vpr activates DNA damage-dependent cellular pathways by directly causing alterations in the structure or the integrity of DNA has not been demonstrated.

Bartz *et al.* (32) showed that Vpr-induced G_2 arrest is independent of ATM function. In addition, p53, which is associated with key aspects of the DNA damage response, is not necessary for the *vpr*-mediated cell cycle arrest or apoptosis (24). In this work, we examined the potential involvement of the DNA dam-

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[1] The abbreviations used are: HIV, human immunodeficiency virus; LTR, long terminal repeat; GFP, green fluorescent protein; RNAi, RNA interference; siRNA, small, interfering RNA.

age-signaling pathway that is initiated by ATR in *vpr*-induced G_2 arrest.

EXPERIMENTAL PROCEDURES

Cell Lines—Human cervical cancer cell line HeLa and transformed human embryonic kidney cell line HEK293T were grown in Dulbecco's modified Eagle's medium 10% fetal bovine serum. Human SV40 transformed fibroblasts GM847/ATRkd (a generous gift of Dr. Cimprich, Stanford University and Dr. Handeli, University of Washington) and human osteosarcoma-derived U2OS ATRkd cell lines were maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum with 400 μg/ml G418 and 200 μg/ml hygromycin B.

Plasmids—We described the lentiviral vector, pHR-GFP, in a previous report (33). pHR-VPR was derived by substituting the B7.1 cDNA in place of human T-cell lymphotrophic virus-I tax in the vector pHR′CMV/Tax1/eGFP (34). This was accomplished by digesting pCMV-vpr (19) with *Not*I followed by Klenow treatment and further digestion with *Xho*I. We then ligated the *vpr*-containing band to pHR′CMV/Tax1/eGFP, which was previously digested with *Sal*I, treated with Klenow, and further digested with *Xho*I.

Viral Vector Production and Titration—Lentiviral vectors were produced by transient transfection of HEK293T cells. For defective lentivirus vector production, pHR-GFP and pHR-VPR plasmids were co-transfected with pCMVD8.2ΔVpr (35) and pHCMV-VSVG (36) using calcium phosphate-mediated transfection (27). Virus supernatant was collected at 48, 72, and 96 h post-transfection. Harvested supernatants were cleared by centrifugation at 2,000 rpm and frozen at −80 °C. Vector titers were measured by infection of HeLa cells as described below followed by flow cytometric analysis of cells positive for the reporter molecule, green fluorescent protein (GFP). Vector titers were calculated as Titer = $(F \times C_0 / V) \times D$, where F is the frequency of GFP (+) cells by flow cytometry, C_0 is the total number of target cells at the time of infection, V is the volume of inoculum, and D is the virus dilution factor. Virus dilution factor used for titrations was $D = 10$. Total number of target cells at the time of infection was 10^6.

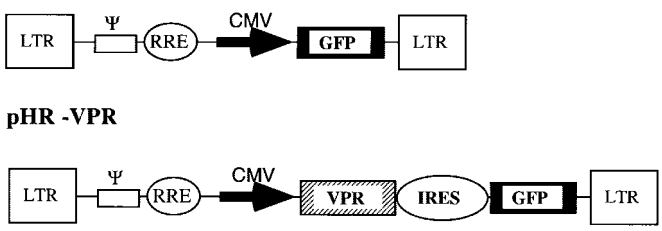

FIG. 1. **Defective lentiviral vectors.** For simplicity, only viral sequences are shown, and bacterial sequences were omitted. Ψ, HIV-1 packaging sequence; *RRE*, rev-responsive element; *CMV*, cytomegalovirus immediate-early promoter; *IRES*, internal ribosome entry site from the encephalomyocarditis virus. Vector production was accomplished by co-transfecting pHR-GFP or pHR-VPR with pCMVΔR8.2ΔVpr and pCMV-VSVG using calcium phosphate-mediated transfection.

Cell Cycle Analysis—Cells were infected with either pHR-VPR or pHR-GFP at a multiplicity of infection of 2.5 to achieve greater than a 90% infection rate was as measured by counting GFP-positive cells. Cells were detached with 2 mM EDTA, washed in phosphate-buffered saline, fixed with 70% ethanol for over 18 h at −20 °C, then stained with propidium iodide solution for 30 min at 4 °C (20 μg/ml propidium iodide, 11.25 kunitz units/ml RNase A in phosphate-buffered saline). If <90% infection was achieved the cells were fixed in 0.25% paraformaldehyde for 1 h to preserve GFP fluorescence, and only the GFP-positive cells were gated to represent the infected fraction of the cells. Flow cytometric analysis was performed in an Epics Elite ESP (Coulter Corp., Hialeah, FL). Cell cycle analysis was performed using Multicycle AV software (Phoenix Flow Systems, San Diego, CA). All cell cycle experiments were performed at least three times, and typical results are shown.

Drug Treatments—LY294002 (Cell Signaling Technology, Beverly, MA) was used at 50 μM. Caffeine (Sigma) was used at 2.5 mM. Doxorubicin (Sigma) was used at 4 μM. Taxol was used at 25 nM. UCN-01 (NSC 638850) was obtained from Developmental Therapeutics Program at the National Cancer Institute and used at 300 nM.

Western Blot—HeLa cells were washed in phosphate-buffered saline and lysed in modified radioimmune precipitation assay buffer (Cell Signaling Research, Beverly, MA). Protein concentration in the lysate was obtained using a modified Lowry method using Bio-Rad protein assay II kit, catalog number 500−002. 100 μg of protein were loaded onto a 10% SDS-PAGE gel and electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were blocked in Tris-buffered saline, 0.2% Tween 20, and 5% nonfat dry milk and probed with monoclonal antibodies directed against Chk1 (1:250 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or Chk1-S345-P (Cell Signaling Technology) followed by a horseradish peroxidase-linked anti-mouse secondary antibody (1:1000 dilution; Amersham Biosciences). Proteins were detected with the use of the enhanced chemiluminescence reagent (Pierce). All Western blots were performed at least three times, and results of a typical experiment are shown.

Luciferase Assays—Transient transfections of U2OS-ATRkd cells for luciferase measurement were performed using electroporation as described previously (27) using 5 μg of either pCMV-Vpr or 5 μg of pCMV-thy and 5 μg of LTR^{HIV-1}-Luc (27). After electroporation, cells were plated at a density of 1×10^6/10 cm dish. At 48 h after the transfection cells were lysed and assayed for luciferase activity with a commercially available luciferase assay kit (Promega Corp.) using a LumiCount microplate reader (Packard Instrument Co.). The luciferase

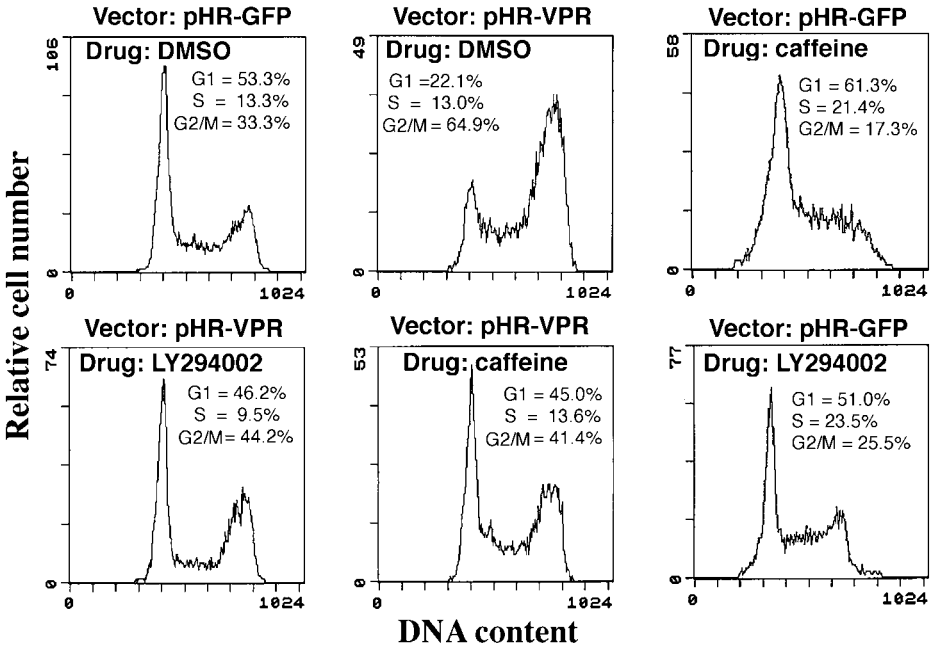

FIG. 2. **Chemical inhibitors of ATM/ATR function block Vpr induced G_2 arrest.** HeLa cells were treated as indicated and infected with either pHR-VPR or pHR-GFP lentivirus vectors. Cell cycle profiles were analyzed by flow cytometry 36 h after infection. *Left peaks* of the histogram charts represents diploid (G_1) populations, whereas the *right peaks* represents tetraploid (G_2/M) population. *DMSO*, Me_2SO.

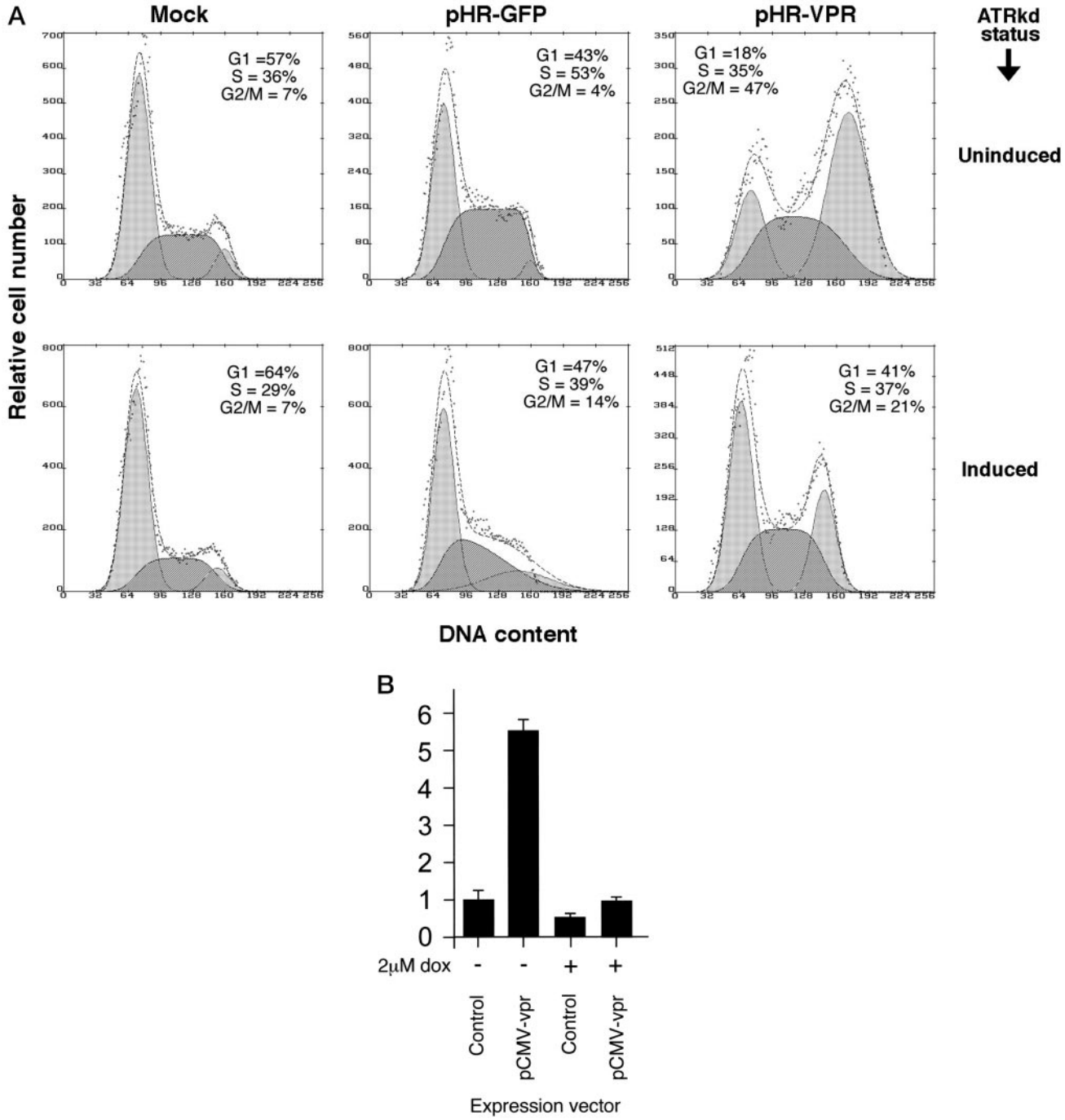

FIG. 3. **Expression of the ATR dominant-negative mutant abrogates Vpr-induced G_2 arrest and transactivation.** *A*, U2OS cells stably transfected with an inducible ATR dominant negative (*ATRkd*) expression cassette were not treated (ATRkd uninduced) or induced (incubated with doxycycline) to express ATRkd. Cells were then either not infected or infected with pHR-GFP or pHR-VPR. Analyzed cell cycle histograms are shown. *B*, U2OS ATRkd cells were treated with 2 μM doxycycline for 48 h to induce ATRkd (*dox+)* or left untreated (*dox−*) and were transiently transfected with either a Vpr-expressing plasmid or a control plasmid. Forty-eight hours after transfection the luciferase activity was measured in the cell lysates. All values were normalized first to total protein content and then to the transactivation value of the control plasmid transfected, *dox−* sample (assigned value of 1). Results represent the mean of three experiments. S.D. are shown as *error bars*.

assay was performed using the following settings: photomultiplier tube = 1,100 V; gain level = 5.0; read length = 0.5 s. Each experiment was performed in triplicate, and each measurement was the average of triplicate readings. Luciferase light units were normalized to 1 mg of protein content (Bio-Rad).

RNAi-mediated Knockdown—RNAi-mediated knockdown of Chk1 was performed as described in (37). ATR RNAi-mediated knockdown was performed as described in Casper *et al.* (14). The 19-nucleotide targets for short, interfering RNAs (siRNAs) are as follows: RNAi for ATR 9DS, 5′-AAC CTC CGT GAT GTT GCT TGA-3′ (RNAi2; catalog number P-002090; Dharmacon, Boulder, CO); Chk1, 5′-AAG CGT GCC GTA GAC TGT CCA-3′ (cat number P-002076; Dharmacon); control non-silencing RNAi, 5′-AAT TCT CCG AAC GTG TCA CGT-3′ (catalog number 80–1130; Qiagen, Valencia, CA). RNAi transfection efficiency was estimated by the percentage of fluorescence-positive cells in the control transfections with a fluorescein isothiocyanate-conjugated oligonucleotide. The efficiency of oligonucleotide transfection was always equal or higher than 80%.

RESULTS

ATR Function Is Required for Induction of Vpr-induced G_2 Arrest and Transactivation—To begin to probe the potential role of ATR in HIV-1 vpr-induced G_2 arrest we first used the

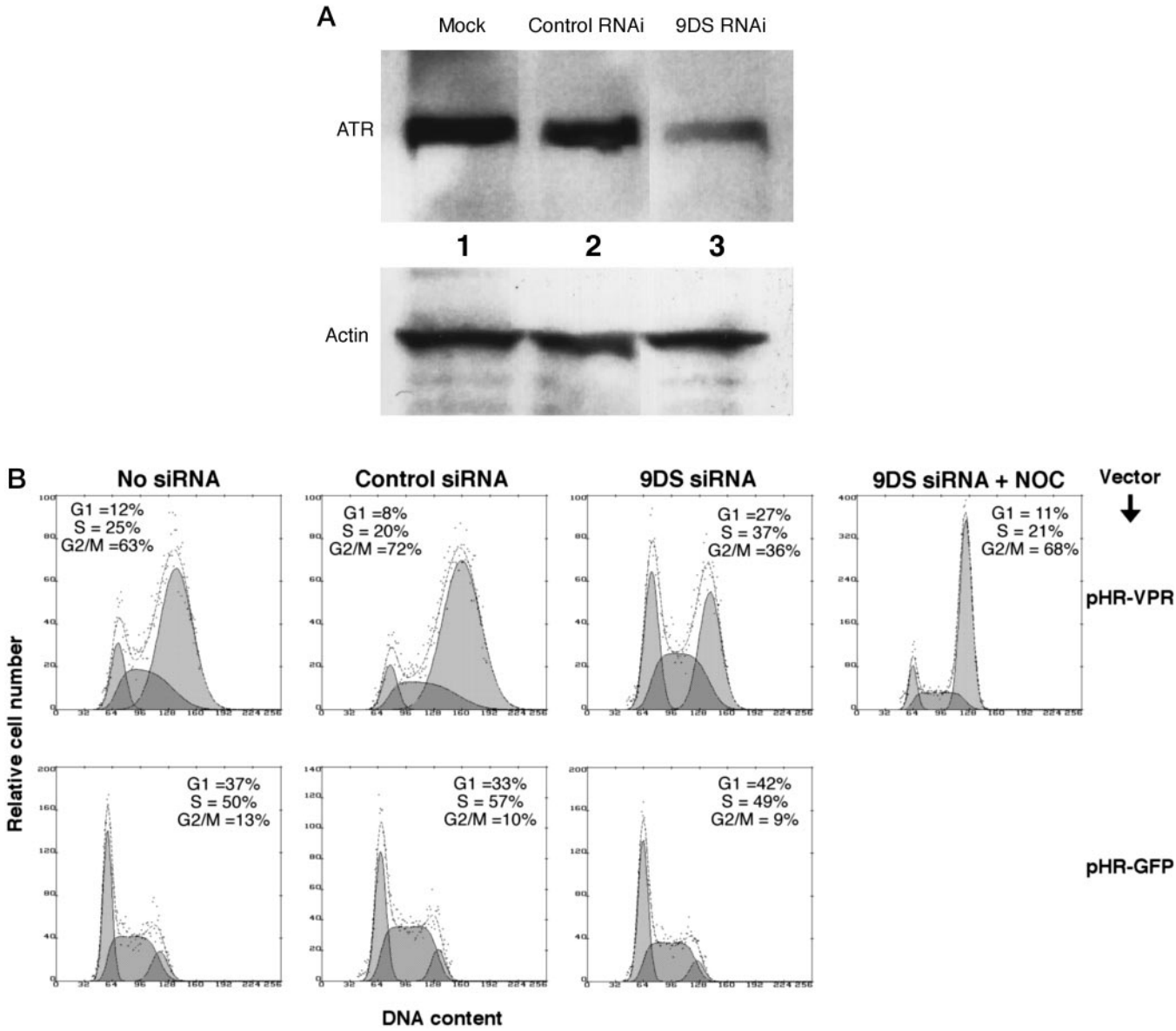

FIG. 4. **ATR knockdown by RNAi reduces Vpr-induced G_2 arrest.** *A*, immunoblot with ATR (*upper panel*)- or actin (*lower panel*)-specific antibodies. HeLa cells were either not transfected (no oligo; *lanes 1*) or transfected with a control double-stranded RNA (*lanes 2*) or an siRNA specific for ATR, 9DS (*lanes 3*). *B*, cell cycle profiles of RNAi-treated cells. Parallel samples as above were transduced with either pHR-GFP (*lower panels*) or pHR-VPR (*upper panels*). Cell cycle analysis was performed by flow cytometry, and analyzed cell cycle histograms are shown. *NOC*, nocodazole.

pharmacological inhibitor of phosphatidylinositol 3-kinase-like protein kinases, LY294002 (38, 39). HeLa cells were treated with either 50 μM LY294002 in Me_2SO or with Me_2SO alone and then infected with either of the lentivirus vectors, pHR-VPR or pHR-GFP (Fig. 1). These vectors encode either Vpr and GFP as a marker (pHR-Vpr) or GFP alone (pHR-GFP) and are packaged and titrated as described under "Experimental Procedures." Infection with pHR-Vpr, but not pHR-GFP, induces detectable G_2 arrest at 24 h post-infection and maximal G_2 arrest between 36 and 48 h when it reaches a plateau.[2] We analyzed the cell cycle profiles of infected cells at 36 h after infection using flow cytometry (Fig. 2). The addition of LY294002 or caffeine (27) largely alleviated the Vpr-induced G_2 arrest. The addition of LY294002 or caffeine to pHR-GFP-transduced cells did not significantly affect their cell cycle profiles.

In a previous study, Bartz *et al.* (32) tested whether ATM −/− (AT) cell lines were able to arrest in response to Vpr. Bartz *et al.* (32) demonstrated that AT cells transfected with *vpr* arrest in G_2 with indistinguishable kinetics from ATM +/+ cells. The results obtained using inhibitors taken together with those using ATM −/− cells suggest that a phosphatidylinositol 3-kinase-like protein other than ATM, presumably ATR, may be the mediator of Vpr-induced G_2 arrest. Therefore, we decided to use more direct experiments to test the role of ATR.

A kinase deficient ATR that carries an Asp-2475 to Ala mutation within the catalytic domain of the protein was previously described (15, 16). This ATR mutant, termed ATRkd (kinase-deficient), is defective in autophosphorylation and, when expressed in mammalian cells, acts as a dominant-negative regulator of wild-type ATR. U2OS7/ATRkd is a human osteosarcoma cell line that was stably transduced with a tetracycline-inducible ATRkd construct (16, 40).

We utilized the U2OS/ATRkd cells to further investigate the role of ATR in Vpr-induced G_2 arrest (Fig. 3, *panel A*). Expression of ATRkd was induced by the addition of 2 μM doxycycline

[2] M. Roshal, B. Kim, Y. Zhu, P. Nghiem, and V. Planelles, unpublished observations.

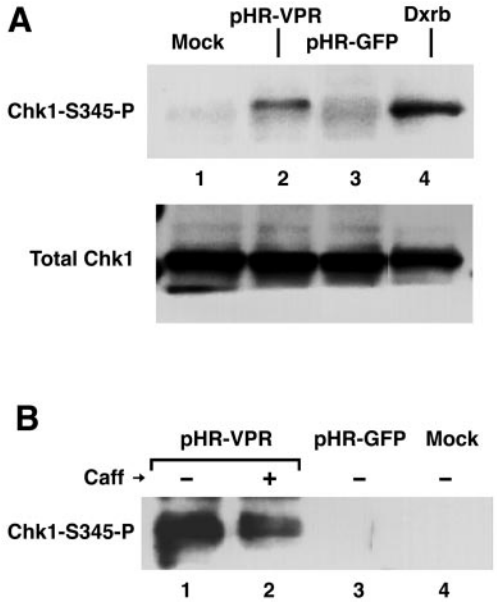

FIG. 5. **Vpr induces phosphorylation of Chk1 at Ser-345 that can be inhibited by caffeine treatment.** *A*, cells were mock-infected, infected with indicated lentivirus vectors, or treated with doxorubicin, lysed, and analyzed for Chk1 phosphorylation using an Ser-345-specific phospho-antibody. *B*, parallel samples were treated as in *A* except in the presence or absence of 2.5 mM caffeine, as indicated.

for 48 h. After doxycycline induction, the cells were infected with either pHR-VPR or pHR-GFP. Forty-eight hours after infection, we examined the cell cycle profiles of the infected cells. Uninduced U2OS/ATRkd cells displayed a normal cell cycle profile when not infected (Mock) or infected with pHR-GFP and displayed G_2 arrest when infected with pHR-VPR. Therefore, U2OS/ATRkd cells, in the absence of ATRkd induction, are sensitive to the cytostatic effect of Vpr. After induction of ATRkd expression with doxycycline, mock-infected cells displayed a normal cell cycle profile. However, under induction conditions, U2OS/ATRkd cells were significantly less sensitive to Vpr-induced G_2 arrest. To rule out the possibility that our observations may be specific to U2OS cells, we used an additional ATRkd-inducible cell line, GM847 (15). Similar to U2OS/ATRkd cells, GM847-ATRkd cells also became resistant to Vpr-induced G_2 arrest upon ATRkd expression (data not shown). U2OS cells contain wild-type p53 whereas GM847 cells are transformed with SV40 large T antigen, which blocks p53 function. Consistent with prior observations (24), the p53 status of the cells does not appear to influence Vpr-induced G_2 arrest.

Viruses typically manipulate the host cell biology to ultimately benefit their propagation. The ability of HIV-1 to induce G_2 arrest through expression of *vpr* provides at least one known benefit to virus replication; that is, an increase in the transcriptional activity of the LTR. Numerous studies conclude that *vpr* acts as a moderate transactivator (26, 27, 29, 41, 42). The ability of Vpr to transactivate the viral promoter is related to the fact that the LTR has features of a G_2-responsive promoter. For example, induction of G_2 arrest with genotoxic agents provides a similar level of transactivation, as does *vpr* expression. Incubation of caffeine, a drug that alleviates DNA damage-dependent G_2 arrest, abrogates *vpr*-induced transactivation. We reasoned that if activation of ATR is the cause of *vpr*-induced transactivation and is the relevant target of caffeine-mediated transactivation reduction, then expression of ATRkd should inhibit such transactivation. To test the previous hypothesis, U2OS/ATRkd cells were treated with either control medium or 2 μM doxycycline for 48 h to induce ATRkd (Fig. 3, *panel B*). These cells were then transfected with pLTR^{HIV-1}-luciferase (a reporter construct in which luciferase is expressed under the control of the LTR) and either a *vpr* expression vector (pCMV-VPR) or a control expression vector expressing Thy-1 marker (pCMV-thy). Forty-eight hours after transfection, cells were lysed, and the luciferase activity was measured and normalized to the protein content of the lysates. All values were normalized first to protein content and then to the transactivation value of the control plasmid transfected in the doxycycline-minus (uninduced) treatment (assigned a value of 1; Fig. 4, *panel B*). The presence of ATRkd was concomitant with abrogation of the ability of *vpr* to increase the LTR transactivation.

Knockdown of ATR Leads to a Decrease in Vpr-induced G_2 Arrest—Although overexpression of ATRkd has been used to study ATR function, it remains formally possible that the presence of ATRkd, the dominant-negative mutant, affects the function of proteins other than ATR and, therefore, modulates other unsuspected checkpoint proteins. To induce inactivation of ATR by an independent method, we utilized RNAi. RNAi is a recently described mechanism utilized by eukaryotic cells to down-regulate the steady-state levels and/or the translation of specific mRNAs (43–45). RNAi is accomplished by short (21–22 nucleotide) double-stranded RNA oligonucleotides (siRNAs) that are specific for the targeted mRNA.

We targeted ATR by transfecting synthetic, duplex RNA oligonucleotides as described under "Experimental Procedures." As controls we used an oligonucleotide duplex containing a nonspecific sequence and mock-transfection (no oligonucleotide). Detection of ATR protein by Western blot was performed on samples taken 72 h after the initial transfection (Fig. 4, *panel A*). Densitometry scanning demonstrated a relative ATR protein down-regulation of 70% when using 9DS (ATR-specific) RNAi duplex when compared with the mock transfection. The transfection with control, nonspecific siRNA did not change ATR protein levels. Transduction of Vpr in 9DS-transfected cells either 48 (data not shown) or 72 h after the RNAi transfection yielded a significantly attenuated G_2 arrest when compared with either HeLa cells transfected with nonspecific sequence or untransfected cells (Fig. 4, *panel B*). To rule out the possibility that this observation is due to specific depletion of the G_2 cells, we treated the ATR-transfected, Vpr-transduced cells with the M-phase-arresting drug, nocodazole. We reasoned that nocodazole would retain in M those cells that were allowed to leave G_2 due to the ATR knockdown. However, if cells treated with ATR siRNA were dying in G_2, treatment with nocodazole would not prevent such death. Treatment with nocodazole caused accumulation of the cells at the G_2/M boundary. This observation demonstrates that in the absence of ATR function, Vpr-transduced cells are capable of entering mitosis.

Vpr Induces Chk1 Phosphorylation—Chk1 is a direct target for ATR in response to DNA damage. When cells sense DNA damage, ATR phosphorylates Chk1 on Ser-345 resulting in increased Chk1 activity. We wished to ascertain whether Vpr-induced ATR activation would also result in phosphorylation of Chk1 on Ser-345. HeLa cells were infected with either pHR-VPR or pHR-GFP. Thirty-six hours post-infection, we analyzed the phosphorylation status of Chk1 by Western blot using a Ser-345-specific phospho-antibody (Fig. 5). Mock-infected (Fig. 5, *panel A*; *lane 1*) and pHR-GFP-infected (Fig. 5, *panel A*; *lane 3*) cells only revealed faint bands corresponding to Chk1-S345-P. However, cells infected with pHR-VPR (Fig. 5, *panel A*; *lane 2*) or treated with doxorubicin (Fig. 5, *panel A*; *lane 4*) displayed a significant amount of Chk1-S345-P. Inhibition of ATR and ATM function by incubating cells with caffeine resulted in a significant (60% reduction by densitometry scanning), although not complete decrease of Chk1 phosphorylation (Fig. 5, *panel B*; *lanes 1* and *2*).

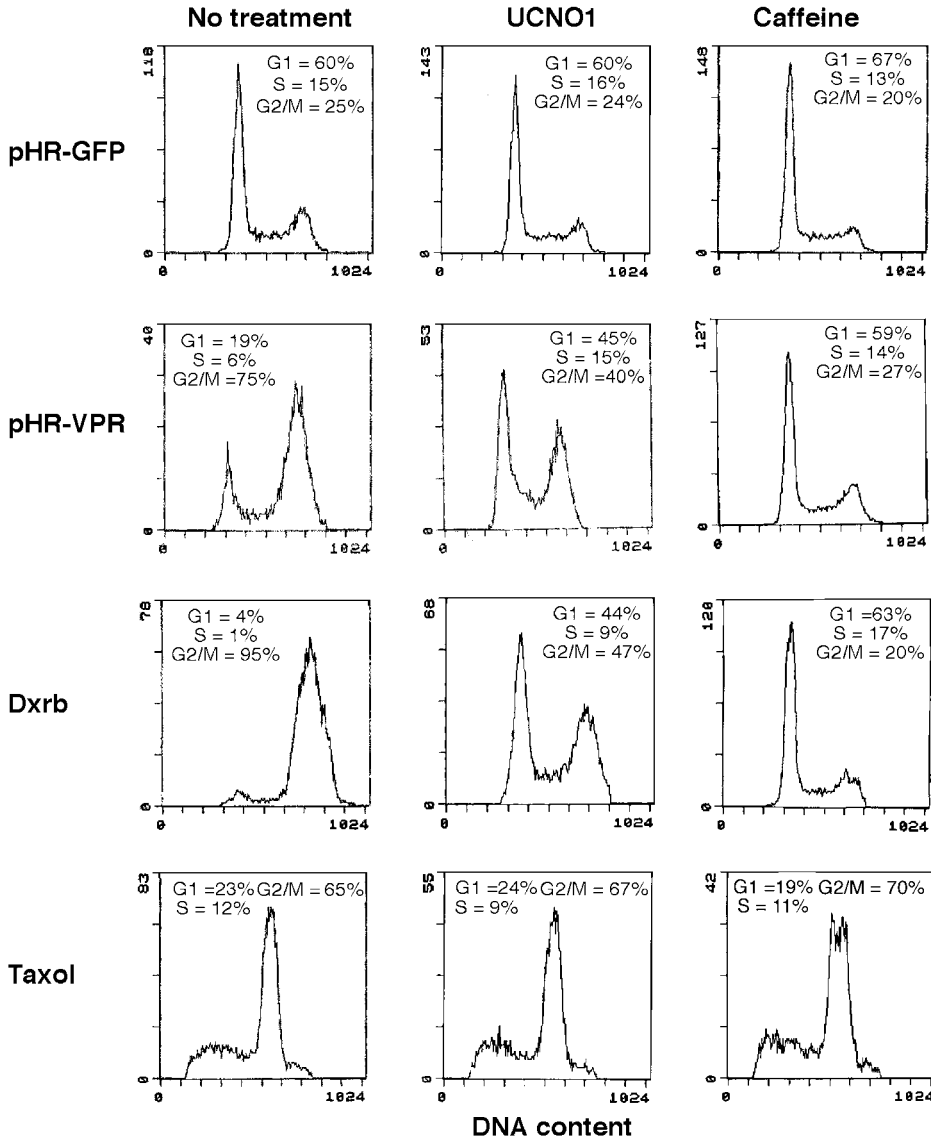

FIG. 6. **Pharmacological inhibition of Chk1 and related kinases results in inhibition of the Vpr-induced G_2 arrest.** Cell cycle analysis of HeLa cells in the absence of treatment (*left panels*) or treated with UCN-01 (*middle panels*) or caffeine (*right panels*) and infected with indicated lentivirus vectors or treated with doxorubicin or taxol. *Dxrb*, doxorubicin.

Inhibition of Chk1 and Related Kinases with UCN-01 Relieves Vpr-induced G_2 Arrest—UCN-01 is a radiosensitizing agent that targets Chk1 as well as the related kinases Chk2 and c-Tak (46). We hypothesized that inhibition of Chk1 by UCN-01 would result in reduction of G_2 arrest-induced by Vpr. To test this hypothesis, we treated HeLa cells with 200 nM UCN-01 (the concentration that is sufficient to completely inhibit Chk1 and c-TAK but not Chk2 (46)). Incubation with UCN-01 resulted in reduction of Vpr-induced G_2 arrest (Fig. 6), and consistent with previous observations (47), it also reduced doxorubicin-induced G_2 arrest. Caffeine appeared to more effectively relieve the cell cycle block than UCN-01 for both Vpr and doxorubicin. As a negative control for alleviation of cell cycle arrest, we used taxol treatment. Taxol-treated cells arrest after entry into mitosis due to inability of microtubules to initiate chromosome separation (48). Therefore, taxol induces a type of arrest that is not enforced by ATR or Chk1 and, therefore, should not be relieved by caffeine or UCN01. As expected neither UCN-01 nor caffeine had an effect on taxol-mediated cell cycle arrest.

Knockdown of Chk1 by RNAi Results in Reduction of Vpr-induced G_2 Arrest—Because UCN-01 is not absolutely specific for Chk1 (46), we were unable to conclude that Chk1 is necessary for induction of G_2 arrest by Vpr. We addressed this question by performing knockdown of Chk1 by RNAi. A recent report indicated that using RNAi to target Chk1 results in a high level of Chk1 protein level knockdown and partial abrogation of Chk1-dependent checkpoint (37). Using synthetic, duplex RNA oligonucleotides as described by Zhao *et al.* (37), we achieved a 90% decrease in Chk1 protein level in HeLa cells (Fig. 7; *panel A*) as evidenced by Western blot. To test whether Chk1 knockdown would result in attenuation of the Vpr-induced G_2 arrest, we proceeded to infect the above RNAi-targeted cells with either pHR-VPR or pHR-GFP and analyzed the resulting cell cycle profiles (Fig. 7; *panel B*). Cells treated with Chk1 siRNA demonstrated a significant reduction of G_2 arrest when compared with cells treated with no duplex or control duplex. Cells treated with Chk1-specific siRNA did not display appreciable changes in cell cycle profile when mock-infected (Fig. 7; *panel B*) or when infected with pHR-GFP (data not shown). Therefore, Chk1 is necessary for the induction of G_2 arrest by *vpr*, and its role is consistent with the notion that Chk1 is a target of ATR (1).

DISCUSSION

In the present study we investigated the potential role ATR in the biology of Vpr. Using pharmacological agents, a dominant-negative mutant, and RNA interference we have demon-

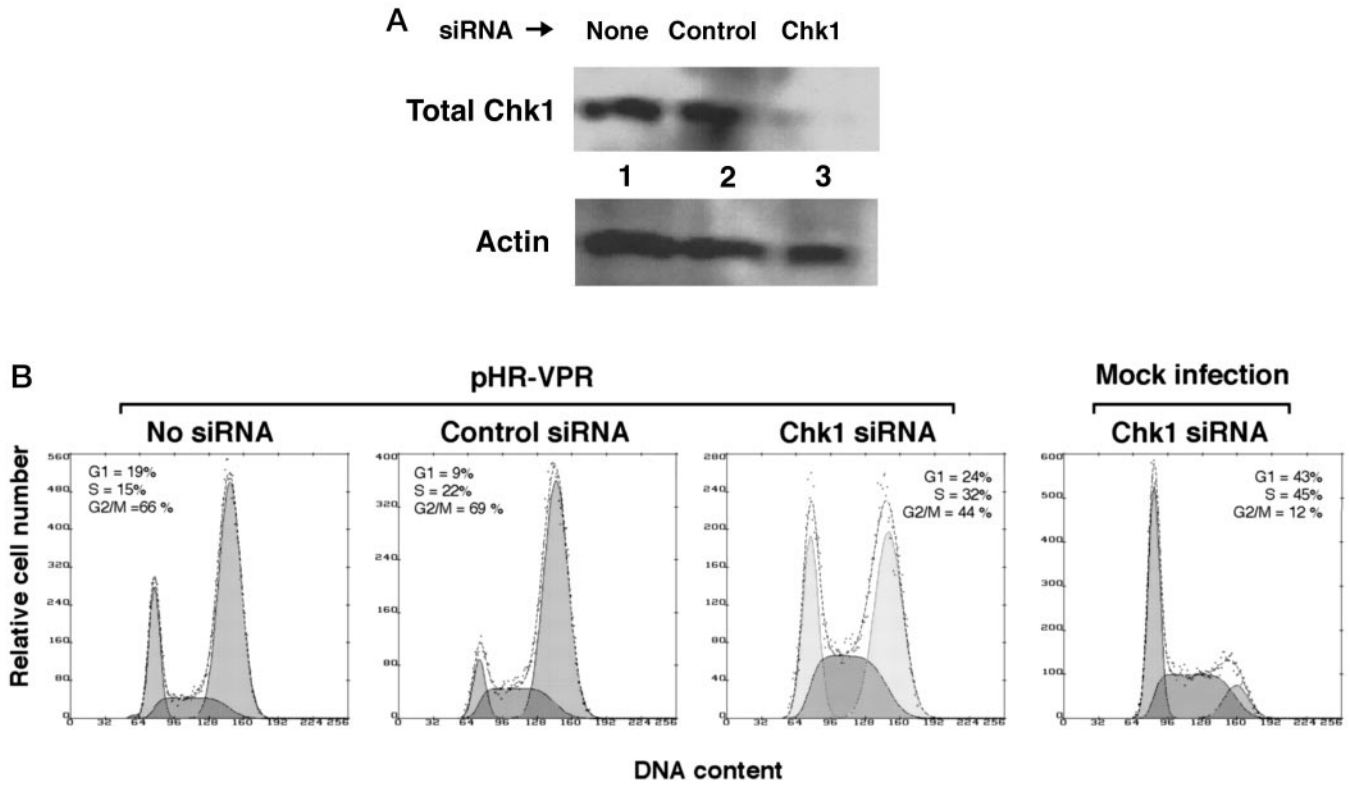

FIG. 7. **RNAi knockdown of Chk1 reduces Vpr-induced G_2 arrest.** *A*, immunoblot of total Chk1 (*upper panel*) or actin (*lower panel*). HeLa cells were either mock-transfected (no oligo; *lane 1*) or transfected with control siRNA (*lanes 2*) or with siRNA specific for Chk1 (*lane 3*). *B*, cell cycle analysis of mock-transfected (no siRNA) cells or cells transfected with control siRNA or Chk1-specific siRNA that were either infected with pHR-VPR or mock-infected.

strated that ATR activity is required for full induction of the Vpr-induced G_2 arrest. The events after activation of the ATR pathway by Vpr closely parallel those observed upon activation of the DNA damage checkpoint controlled by ATR. These events include phosphorylation of Chk1 and hyperphosphorylation of Cdc2. These observations suggest that the regulation of the transition between G_2 and M by Vpr is similar to that induced by DNA-damaging agents that specifically activate ATR.

It is not known whether Vpr actually causes DNA damage or whether, alternatively, it generates a signal that "mimics" DNA damage by activating one of the DNA damage sensors. Our previous observations suggest a possible difference between the DNA damage and Vpr-induced checkpoint activation pathways (27). Inhibition of the checkpoint proteins in the context of DNA damage usually results in increased apoptosis. Our earlier observations demonstrated that inhibition of the Vpr-induced checkpoint by caffeine resulted in unexpectedly decreased apoptosis (27).

The ATR and Chk1 knockdown experiments we present produced incomplete relief of the Vpr-induced G_2 arrest. This observation could be explained by the existence of an alternative or complementary signaling pathway that can also be activated by Vpr to induce G_2 arrest. An alternative, simpler explanation would be based on the fact that RNAi knockdowns were not complete (70% for ATR and 90% for Chk1). In light of the fact that pharmacological inhibition of either ATR with caffeine completely relieved the cell cycle block by Vpr, it appears more likely that the second explanation is true.

Previously, attempts have been made to study Vpr-biology in the fission yeast. In that system neither the knockout of the ATR/ATM homologue, Rad3, nor knockouts of the Chk1 and Chk2 homologues resulted in a reduction of the Vpr-induced growth defect (49). It is possible that an alternative DNA damage-responsive system is activated in the fission yeast. Rad3 mutants in fission yeast are viable (50), whereas the mec1 mutations in the budding yeast and ATR in human cells are lethal. It appears that mammalian ATR plays a role in patrolling for genomic integrity during normal replication. ATR deletion leads to apoptosis-independent chromosomal breakdown and expression of fragile sites (13, 14). Rad3 deletion in *Schizosaccharomyces pombe*, however, does not compromise genomic integrity in the absence of exogenously induced genotoxic stress. Moreover in mammalian cells, ATR/ATM-mediated Chk1 activation requires breast cancer susceptibility gene-1 (BRCA1), whereas homologues are not known in yeast (51). This suggests that there is an incomplete functional homology between the fission yeast Rad3 and the ATR signaling systems that may account for the observed differences.

A recent report (52) demonstrates that HIV-1 Vpr induces defects in nuclear lamin structure and consequent nuclear herniation with chromatin structure alterations. Vpr-induced changes in chromatin structure may lead to stalled DNA replication. ATR has recently emerged as a key sensor of incomplete replication status of mammalian cells (7, 53–55). Therefore, the model proposed by De Noronha *et al.* (52) and the results presented here would be consistent with a scenario where Vpr causes nuclear herniations that slow down DNA synthesis, which in turn activates ATR.

In view of the above findings we propose the following model for the signaling induced by the Vpr. Via interaction with lamins and the subsequent nuclear herniation, Vpr induces alterations in the chromatin structure that lead to stalled replication. The alterations in chromatin structure and replication are sensed by ATR, which in turn activates Chk1. Further activation of the ATR/Chk1 cascade leads to inhibition of Cdc2, the key regulator of the G_2/M transition. Likely candidates as the immediate inhibitors of Cdc2 may be Cdc25C (21) and Wee1 (56, 57).

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