JOURNAL OF VIROLOGY, Dec. 1997, p. 9732–9742 Vol. 71, No. 12
0022-538X/97/$04.00+0

Human Immunodeficiency Virus Type 1 Vpr Interacts with HHR23A, a Cellular Protein Implicated in Nucleotide Excision DNA Repair

ELIZABETH S. WITHERS-WARD,[1] JEREMY B. M. JOWETT,[1] SHEILA A. STEWART,[1] YI-MING XIE,[1] ALAN GARFINKEL,[2] YOSHIO SHIBAGAKI,[3] SAMSON A. CHOW,[3] NEIL SHAH,[1] FUMIO HANAOKA,[4] DAVID G. SAWITZ,[5] ROBERT W. ARMSTRONG,[5] LARRY M. SOUZA,[5] AND IRVIN S. Y. CHEN[1]*

Departments of Microbiology and Immunology and Medicine,[1] Departments of Medicine, Cardiology, and Physiological Science[2] and Department of Molecular Medical Pharmacology,[3] UCLA School of Medicine, Los Angeles, California 90095-1678; Departments of Molecular and Cellular Biology, Osaka University, Suita-shi, Osaka 565, Japan[4]; and Amgen, Thousand Oaks, California 91320[5]

Received 12 June 1997/Accepted 12 September 1997

The human immunodeficiency virus type 1 (HIV-1) *vpr* gene is an evolutionarily conserved gene among the primate lentiviruses HIV-1, HIV-2, and simian immunodeficiency viruses. One of the unique functions attributed to the *vpr* gene product is the arrest of cells in the G_2 phase of the cell cycle. Here we demonstrate that Vpr interacts physically with HHR23A, one member of an evolutionarily conserved gene family involved in nucleotide excision repair. Interaction of Vpr with HHR23A was initially identified through a yeast two-hybrid screen and was confirmed by the demonstration of direct binding between bacterially expressed recombinant and transiently expressed or chemically synthesized protein products. Visualization of HHR23A and Vpr by indirect immunofluorescence and confocal microscopy indicates that the two proteins colocalize at or about the nuclear membrane. We also map the Vpr-binding domain in HHR23A to a C-terminal 45-amino-acid region of the protein previously shown to have homology to members of the ubiquitination pathway. Overexpression of HHR23A and a truncated derivative which includes the Vpr-binding domain results in a partial alleviation of the G_2 arrest induced by Vpr, suggesting that the interaction between Vpr and HHR23A is critical for cell cycle arrest induced by Vpr. These results provide further support for the hypothesis that Vpr interferes with the normal function of a protein or proteins involved in the DNA repair process and, thus, in the transmission of signals that allow cells to transit from the G_2 to the M phase of the cell cycle.

Human immunodeficiency virus type 1 (HIV-1) is a causative agent of AIDS. In addition to the retroviral structural genes *gag*, *pol*, and *env*, the HIV-1 genome encodes several accessory genes that are important for replication and/or pathogenesis. One of these genes, *vpr*, encodes an 11-kDa basic protein that appears to serve a number of functions in the viral life cycle. Vpr is incorporated into the virion (7, 49, 50). The incorporation of Vpr into virions requires the p6 domain of the $p55^{gag}$ precursor protein (24, 27, 31, 32, 38). Nuclear localization signals present within both Gag and Vpr are required to assist in the translocation of the pre-integration complex into the nuclei of nondividing cells (5, 6, 20), although the contribution of Vpr to this process has been disputed (13). Both the presence of Vpr in the virion and its function in nuclear import account for the requirement of Vpr for efficient replication of HIV-1 in nondividing cell types such as macrophages (2, 9, 20).

One particularly noteworthy phenotype of the expression of Vpr in cells is the arrest of cells in the G_2 phase of the cell cycle (19, 23, 42, 43). Vpr has been shown to be both necessary and sufficient to cause accumulation of human lymphoid and nonlymphoid cells in the G_2 phase of the cell cycle. Vpr can induce cell cycle arrest both following transfection of expression constructs encoding Vpr as well as in the context of HIV-1 infection with a wild-type *vpr* gene. Vpr-arrested cells have a 4N content of DNA and no mitotic indices are present. In addition, the major regulator of the transition between G_2 and M, Cdc2 kinase, is hyperphosphorylated and exhibits a decreased activity, although Vpr does not appear to directly inhibit Cdc2 kinase activity (3, 19, 23, 42). The ability of Vpr to influence the cell cycle likely relates to an earlier observation that Vpr caused differentiation of rhabdomyosarcoma cells (29). The cell cycle arrest mediated by Vpr also induces growth arrest in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (33, 52). In *S. pombe* the Vpr-induced growth arrest was characterized as arrest at G_2 and correlated with increased phosphorylation of Cdc2 kinase (52). The similarity of these results to those observed in human cells suggests that Vpr disrupts a highly conserved cellular process.

Several other effects have been attributed to Vpr although their significance in viral replication is not well understood. Vpr has been shown to increase the activity of a variety of promoters including the HIV-1 long terminal repeat (8). Vpr isolated from the serum of infected individuals has been shown to activate virus expression in latently infected cell lines and resting peripheral blood lymphocytes of infected individuals (29, 30).

Members of the HIV-2 and SIV_{MAC} families encode *vpr* and an additional gene, termed *vpx*. *vpr* and *vpx* share considerable sequence similarity and are thought to have arisen as a result of a gene duplication event (45). Although *vpr* and *vpx* share significant homology, their nuclear localization and G_2 arrest properties are segregated in the HIV-2 and SIV_{MAC} groups: Vpr mediates G_2 arrest and Vpx is necessary for nuclear localization of preintegration complexes in nondividing cells (12, 39). In the simian immunodeficiency virus (SIV) model, the presence of Vpr increases the viral load and rate of progression to AIDS and appears to confer a selective advantage to the virus (26). Other studies have indicated that mutation of either

* Corresponding author. Phone: (310) 825-4793. Fax: (310) 794-7682.

vpr or *vpx* has little effect on pathogenicity but that loss of both genes results in severe attenuation (14, 21).

Several cellular proteins have been reported to be associated with Vpr including a 41-kDa cytosolic protein that complexes with the glucocorticoid receptor protein (41), an unidentified 180-kDa protein (51), Sp1 (48), TFIIB (1) and uracil DNA glycosylase (4). The roles of these proteins in the various functions attributed to Vpr are not clear.

Vpr-mediated cell cycle arrest is similar in some respects to the arrest of cells at the G_2 phase of the cell cycle induced by damage to DNA. Certain DNA-damaging agents such as nitrogen mustard (HN_2) also arrest cells in G_2 and have an inactive Cdc2 kinase (36, 37). We have recently shown that Vpr-induced G_2 arrest can be alleviated by chemical agents known as methylxanthines, which are known to alleviate the arrest induced by DNA damage (40). Thus, we propose that Vpr may interact with components of the cellular DNA repair pathway that, when activated, signal cells to arrest at the G_2 checkpoint of the cell cycle.

In order to further elucidate the pathways utilized by Vpr to arrest cells in G_2, we used the yeast two-hybrid system to identify human proteins which are potentially capable of physically associating with Vpr. We isolated a partial cDNA encoding one of the human homologs of the *S. cerevisiae* Rad23 protein, HHR23A, a protein thought to play a role in DNA repair. We demonstrate that full-length HHR23A protein when transiently expressed in HeLa cells interacts physically with a bacterially expressed recombinant GST/Vpr fusion protein (GSTVpr). Indirect immunofluorescence and confocal microscopy indicate that the two proteins colocalize within the same subcellular region, principally at or about the nuclear membrane. We also map the Vpr-binding domain in HHR23A to the carboxy-terminal region of the protein and demonstrate that a chemically synthesized peptide representing the C-terminal 45 amino acids (aa) of HHR23A binds to GSTVpr. Significantly, we demonstrate that overexpression of HHR23A in cells leads to partial alleviation of the G_2 arrest induced by Vpr.

MATERIALS AND METHODS

Yeast strains and media. The yeast strain used in the two-hybrid screen, HF7c (10), was grown in rich medium, YPD (1% yeast extract, 2% peptone, 2% dextrose, and 0.02-mg/ml adenine). Transformed strains were grown in complete minimal drop-out medium with the appropriate supplements (1.6 g of complete minimal drop-out powder [BIO 101, La Jolla, Ca]/liter, 0.17% yeast nitrogen base lacking amino acids, 0.5% $(NH_4)_2SO_4$, 2% dextrose, and 20 mg of adenine/ml). Yeast strains were grown at 30°C.

Yeast two-hybrid screen. To identify proteins that interact with HIV-1 Vpr, the complete coding sequence of the HIV-1_{NL4-3} *vpr* gene was amplified by PCR and ligated into the *Nco*I and *Bam*HI sites of the yeast Gal4DB plasmid pAS2 to create the plasmid pAS2Vpr. The sequence of the forward or sense primer used for PCR amplification was 5′-AGATCCCATGGAGATGGAACAAGCCCCAGAAGAC-3′. The sequence of the reverse or antisense primer used for PCR amplification was 5′-AGATGGATCCCTAGGATCTACTGGCTCCATTTCTTG-3′. These primers contain *Nco*I and *Bam*HI sites (underlined) at their 5′ ends for cloning purposes. The sequence of the Vpr insert was determined to ensure that the Gal4DBVpr fusion was in the correct frame and to verify the Vpr coding sequences. The primer used for sequencing was derived from the Gal4 DNA binding domain (Gal4DB) (5′-CAGCATAGAATAAGTGCGAC-3′). The yeast reporter strain HF7c was transformed with pAS2Vpr, and transformants were plated on synthetic drop-out medium lacking tryptophan. A Trp^+ colony, designated B4-2, was further characterized as described below and used in the two-hybrid screen. pAS2Vpr DNA was recovered from B4-2, and both strands of the Vpr insert were sequenced by using the Gal4DB primer and a primer derived from the yeast alcohol dehydrogenase I (AdhI) terminator sequence (5′-GCCGGTAGAGGTGTGGTC-3′). Protein lysates were prepared from overnight cultures of B4-2 and analyzed by Western blotting. We detected a fusion protein of the expected size: approximately 32 kDa. We confirmed that the Gal4DBVpr fusion protein did not activate the *lacZ* reporter gene by testing B4-2 in the β-galactosidase assay described below.

B4-2 was transformed with a yeast expression library that directs expression of fusion proteins between the Gal4 transcriptional activation domain (Gal4AD) and HeLa cDNA-encoded proteins (18). Transformants were plated on synthetic drop-out medium lacking histidine, leucine, and tryptophan. Approximately 10^4 transformants were plated on each of 59 plates and incubated at 30°C for 5 to 10 days. His^+ Leu^+ Trp^+ colonies were then patched onto synthetic drop-out medium lacking histidine, leucine, and tryptophan and containing 2.5 mM 3-amino-(1,2,4)triazole (3-AT). Colonies that were able to grow under these selective conditions were then assayed for β-galactosidase activity. Briefly, His^+ Leu^+ Trp^+ 3-AT^+ patches were lifted onto reinforced nitrocellulose membranes, frozen briefly (20 s) in liquid nitrogen, thawed at room temperature (2 min), and overlaid onto Whatman 3MM filters that were saturated with β-galactosidase buffer (120 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 0.27% [vol/vol] β-mercaptoethanol [pH 7.0]) in petri dishes. Filters were incubated at 30°C for 24 h. Of the 600,000 transformants screened, 173 were His^+ Leu^+ Trp^+ 3-AT^+ and expressed β-galactosidase activity. As an additional control in the library screening, the library DNA was omitted in one transformation reaction of B4-2. No His^+ Leu^+ Trp^+ colonies were detected. Plasmid DNA was recovered from positive colonies and introduced into *Escherichia coli* MH4, a *leuB* mutant, by electroporation. Transformants carrying only the library plasmid were selected for by plating on minimal medium. Plasmid DNA was isolated from two Leu^+ colonies from each transformation by using the QIAprep Spin Miniprep kit (QIAGEN Inc., Chatsworth Calif.). Partial sequences of some of the HeLa cDNAs were determined by using a primer derived from the Gal4AD (5′-CCACTACAATGGATGATGTA-3′).

To determine the total number of HHR23A clones isolated in the two-hybrid screen, plasmid DNA isolated from all of the positives identified in the two-hybrid screen was immobilized on nitrocellulose filters and probed with a gel purified ^{32}P-labeled *Eco*RI fragment isolated from the HHR23A(C10) clone. Clones that hybridized with the HHR23A probe were sequenced with the Gal4AD primer to determine the 5′ end of the cDNA insert.

Yeast plasmids containing HHR23A cDNA sequences [HHR23A(B213) and HHR23A(B25)] were tested for target specificity by cotransforming HF7c with the HHR23A(B213) or HHR23A(B25) library plasmid and pAS2 or a Gal4 DBSNF1 fusion protein. The HHR23A library plasmid did not activate either the *his* or *lacZ* reporter genes in the presence of the Gal4DB or the Gal4DBS NF1 fusion. The screening was performed as described above.

Standard yeast methods were used throughout (1a) except where otherwise indicated. DNA sequencing was performed with automated 373A and 377 DNA sequencers and *Taq* DyeDeoxy Terminator cycle sequencing kits (Applied Biosystems).

Mammalian expression constructs. The dual expression plasmids BSVprThy and BSVprXThy (23) contain either Vpr or the C-terminal truncation mutant VprX and the Thy 1.2 coding sequences which are both expressed from tandem copies of the cytomegalovirus immediate-early promoter unit. These vectors also contain simian virus 40 transcription termination sequences and an untranslated intron of the cytomegalovirus immediate-early promoter. The HHR23A(B213) coding sequence (nucleotides [nt] 589 to 1155) was amplified by PCR from the HeLa cDNA library plasmid recovered in the two-hybrid screening. The sequence of the forward primer was 5′-CCGCTCGAGATGGACTACAAAGACGATGACGATAAAAGAGCCAGCTACAACAAC-3′. The sequence of the reverse primer was 5′-CCGACGCGTGGGCTTCGGTGGCCTGGCTTCC-3′. The PCR-amplified fragment was then inserted into the *Xho*I and *Mlu*I sites of the expression plasmid pXC (39) to create pXCB213. The full-length HHR23A coding sequence (nt 40 to 1155) was amplified by PCR from the HeLa cDNA library used in the two-hybrid screening. The sequence of the forward primer was 5′-GAGCCGCTCGAGATGGACTACAAAGACGATGACGATAAAGCCGTCACCATCACGCTC-3′. The sequence of the reverse primer was as described for the construction of pXCB213. The PCR-amplified fragment was inserted into the *Xho*I and *Mlu*I sites of PXC to create pXCR23A. The forward primers contain an *Xho*I site, a translation initiation site, and the M2 FLAG epitope tag (underlined) at their 5′ ends. The reverse primer contains an *Mlu*I site at its 5′ end. The DNA sequences of all of the HHR23A inserts in the plasmids described above were confirmed by sequence analysis. The HHR23A DNA containing a frameshift at aa 346 is designated pXCR23Atrunc.

BSXCHACdc2 was constructed by PCR amplification of the Cdc2 coding sequences in pOB231-Cdc2 (28). The sequence of the forward primer used was 5′-CGGGCTCGAGATG TACCCATACGATG T TCCAGAT TACGCCATGGAAGAT TATACCAAA-3′. The sequence of the reverse primer was 5′-CGGGGATATCCTACATCTTCTTAAT-3′. The PCR-amplified fragment was then inserted into the *Sal*I and *Sma*I sites of pUC19 to create an intermediate vector. The Cdc2 coding sequence was then inserted into the *Pst*I and *Eco*RI sites of the expression plasmid BSXCVpr to create BSXCHACdc2.

Cells. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with calf serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml). At 18 to 24 h prior to transfection, cells were plated in six-well plates at a density of 2×10^5/well. HeLa cells were cotransfected with either BSVprThy or BSVprXThy and a 20-fold molar excess of either pXC, pXCB213, pXCR23A, or pXCR23Atrunc by using Lipofectin (Gibco BRL) as directed by the manufacturer. Forty-eight hours after transfection cells were analyzed by indirect immunofluorescence, confocal microscopy, and flow cytometry as described below.

Indirect immunofluorescence and confocal microscopy. Forty-eight hours after transfection, cells were plated on chamber slides in growth medium. Twenty-four hours later, growth medium was aspirated and cells were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (in PBS) for 15 to 20 min at room temperature, permeabilized with 0.2% Triton X-100 (Sigma) for 10 min at room temperature, and washed with FACS buffer (PBS, 2% fetal calf serum, 0.2% sodium azide). After being washed, cells were incubated with a mouse 12CA5 monoclonal antibody (diluted 1:300 in FACS buffer) for 60 min at 37°C. After several washes with FACS buffer, cells were incubated with a goat-anti-mouse antibody conjugated with phycoerythrin (Molecular Probes, Eugene, Oreg.) for 60 min at 37°C. The dually transfected cells were then stained with a mouse M2 FLAG monoclonal antibody (diluted 1:300 in FACS buffer) (Eastman Kodak Co., Rochester, N.Y.) as described above, followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglobulin G1 antibody (Caltag, San Francisco, Calif.). After being washed in FACS buffer the slides were preserved in mounting medium (2.4 g of gelvatol 20-30 [Sigma] added to 6 g of glycerol. After being mixed, 6 ml of water was added, and the solution was left at room temperature for several hours. After the addition of 12 ml of 0.2 M Tris (pH 8.5), the solution was incubated at 50°C for 10 min, followed by clarification by centrifuging at 5,000 × *g* for 15 min. DABCO [Sigma] was added to 2.5% to reduce fading. The solution was stored at 4°C.) Background staining was controlled for by omission of the incubation step with one or both of the primary antibodies to establish that the FITC-conjugated goat-anti-mouse immunoglobulin G1 antibody did not cross-react with the mouse 12CA5 antibody. In all cases background was negligible.

Images were acquired on a Zeiss confocal microscope (Model LSM 410; krypton-argon laser) with a 40X/1.3 oil immersion lens. Image analysis was done using AVS software on a Silicon Graphics computer. The fluorescence intensity of Vpr was coded linearly into the red half of the spectrum and that of the HHR23A protein was coded linearly into the blue half. Since the images were acquired sequentially at a fixed setting of the confocal microscope they may be superimposed to reveal colocalization. The superimposed images are a graphical representation of the cross-correlation function; where high values of Vpr intensity (red) cross-correlate with high values of HHR23A (blue), the resulting lavender signifies colocalization. Fluorochrome bleed-through was ruled out by imaging each channel through the filter for the other channel: both results were negative.

Flow cytometry. Forty-eight hours after transfection, cells were stained with a monoclonal antibody to the murine Thy1.2 cell surface protein directly conjugated with FITC as previously described (23). After being stained, cells were washed and resuspended in hypotonic buffer containing propidium iodide (PI) (3.4 mM sodium citrate, 0.3% Triton X-100, 0.1-mg/ml PI, 0.02-mg/ml RNase A) (25), incubated on ice for 10 min, and analyzed by flow cytometry using the Lysis II software (Becton Dickson). A total of 5,000 events were acquired for each sample. Gates were set to exclude clumps and cell debris and on the $Thy1.2^-$ and $Thy1.2^+$ populations. The $Thy1.2^-$ and $Thy1.2^+$ populations were distinguished by comparing the staining pattern of Thy1.2-transfected populations to that of a mock-transfected population of cells. After acquisition of 5,000 events an additional 1,000 to 1,500 $Thy1.2^+$ events were acquired for each sample. The G_1 versus G_2/M ratios presented in Fig. 6 are those calculated from the $Thy1.2^+$ cells. The Thy1.2-FITC fluorescence intensity is plotted on the *x* axis and the fluorescence intensity of the PI is plotted on the *y* axis.

Expression of GST fusion proteins in bacteria. The HIV-1_{NL4-3} coding sequence was amplified by PCR and ligated into the *Bam*HI (blunt ended) and *Hin*dIII sites of pGEX-KG (15) to produce pGEXVpr. *E. coli* BL21(DE3) was transformed with the pGEXVpr or pGEX expression plasmid. Cultures (20 ml) were grown overnight at 30°C and were then transferred to 1 liter of growth medium and grown at 30°C to an optical density at 600 nm of 1.0 (2.5 to 3.0 h). Isopropyl-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 5 h of induction at 30°C, cells were harvested and resuspended in PBS (100 ml). Cells were placed on ice, and after the addition of Nonidet P-40 (NP-40) (0.1%), they were disrupted by sonication (500 W) five times (1 min/pulse). Lysates were centrifuged for 60 min (100,000 × *g*) at 4°C. The GSTVpr lysate was dialyzed overnight at 4°C against PBS to increase the yield of purified protein. Glutathione-Sepharose resin (3 ml) which had been pre-washed with PBS was then added to the dialysate or lysate (in the case of glutathione *S* transferase [GST]), and the suspension was rocked gently at room temperature for 30 min. GST proteins bound to glutathione-Sepharose resin were then collected by centrifugation and washed five times (5 min/wash) with PBS (10 ml). After the final wash, the resin was resuspended in PBS (3 ml) and packed into a column. After the column was washed with PBS (10 ml), GST proteins were eluted with 5 ml of glutathione elution buffer (50 mM Tris HCl [pH 8.0], 10 mM glutathione [reduced]). Fractions were collected and analyzed by polyacrylamide gel electrophoresis (PAGE). Those fractions containing GSTVpr protein were pooled and dialyzed against PBS. Protein concentration was determined by the Bradford method. Purified proteins were stored at −70°C.

Ex vivo binding assay. Cell lysates used in the binding assay were prepared in the following manner. Forty-eight hours after transfection cells were lifted from plates and washed once in PBS. Cells were then collected by centrifugation, resuspended in lysis buffer (50 mM NaCl, 25 mM Tris [pH 8.0], 0.5% NP-40, aprotinin [0.007 trypsin inhibitor units], (1.0-μg/ml) leupeptin, and 0.05-mg/ml phenylmethylsulfonyl fluoride), and incubated on ice for 10 to 20 min. After the cell suspensions were passed through a 20-gauge needle five times, the lysates were spun for 20 min at 4°C. Supernatants were transferred into fresh tubes and stored at −20°C. For the binding reactions 30 μl of cell lysate was diluted in binding buffer (50 mM NaCl, 25 mM Tris [pH 8.0]) to a final volume of 250 μl (for a final concentration of 50 mM NaCl, 25 mM Tris [pH 8.0], 0.1% NP-40) and then incubated with 20 μl of glutathione-Sepharose resin, which had been pre-washed in binding buffer that contained 0.1% NP-40, for 30 min at 4°C. Samples were spun briefly, and the supernatants were transferred to fresh Eppendorf tubes. GSTVpr or GST (3 μg) was added to each reaction, and samples were gently rocked at 4°C for 2 to 3 h. Glutathione-Sepharose resin (20 μl) was added to each reaction, and samples were gently rocked for 30 min at 4°C. Samples were washed once in binding buffer that contained 0.1% NP-40 (150 μl) and briefly spun, and all of the wash buffer was carefully removed from the pellet. After the addition of 20 μl of Laemmli buffer, samples were boiled for 5 min and centrifuged in a microcentrifuge at the highest setting for 5 min, and the supernatant (approximately 18 μl) was applied to a 20% polyacrylamide gel and analyzed by Western blotting.

In vitro binding assay. Peptides corresponding to the C-terminal 45 aa of the full-length HHR23A and HHR23B proteins were biochemically synthesized. For detection purposes the peptides were synthesized with a biotin tag on the amino terminus. The aa sequences of the peptides are as follows: HHR23A, N-QEKEAIERLKALGFPESLVIQAYFACEKNENLAANFLLSQNFDDE-C; HHR23B, N-QEKEAIERLKALGFPEGLVIQAYFACEKNENLAANFLLQQ NFDED-C. Peptides were resuspended in purified water (1 mg/ml), and the pH was adjusted to 7.0 with 100 mM NaOH. Aliquots were stored at −70°C and thawed just prior to use. The binding reactions contained 10 μg of GSTVpr or GST and 50 μg of HHR23A or HHR23B peptide in a 200-μl reaction volume of buffer A (20 mM Tris [pH 7.5], 100 mM NaCl, 5 mM dithiothreitol [DTT]). After a 1- to 2-h incubation at 4°C, 100 μl of glutathione-Sepharose was added to each reaction. The glutathione-Sepharose was prepared by washing 0.5 ml of the suspension obtained from the manufacturer three times in buffer B (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM DTT) (1 ml/wash) and then resuspending it in a final volume of 2.0 ml of buffer B. After a 15-min incubation at room temperature, the binding reactions were washed five times (1 ml/wash) in buffer C (20 mM Tris [pH 7.5], 300 mM NaCl, 0.1% NP-40, 1 mM DTT). All of the final wash buffer was carefully removed, and 30 μl of Laemmli buffer was added to the pellets. After being boiled for 5 min, samples were spun in a microcentrifuge for 5 min at the maximum setting, and 8 μl of each reaction was analyzed by PAGE (23% gel) and Western blotting.

The competition experiment was performed as described above with the following exception. After a 2-h incubation of GSTVpr or GST and HHR23A peptide at 4°C, increasing amounts of unbiotinylated HHR23A peptide were added to reactions in a 120-μl volume of buffer A and incubation at 4°C was extended for 1 to 2 h. Ten microliters of each reaction was analyzed by Western blotting.

Western blot analysis. Proteins were separated by PAGE and electroblotted to nitrocellulose membranes. Blots were incubated with mouse monoclonal antibodies specific for either the hemagglutinin or M2 FLAG (Eastman Kodak Co.) epitopes. This was followed by incubation with a horseradish-peroxidase-linked sheep anti-mouse immunoglobulin (Amersham Life Science Inc.). Antibody binding was detected with the ECL detection kit (Amersham Life Science Inc.). In the case of the in vitro binding reactions, biotinylated peptides were detected by incubation with streptavidin conjugated with horseradish peroxidase (Amersham Life Science Inc.).

RESULTS

Identification of cellular proteins interacting with Vpr through a yeast two-hybrid screen. We utilized a yeast two-hybrid screen to identify cDNAs encoding human proteins capable of interacting with HIV-1 Vpr (11). To create the target for the two-hybrid screen we ligated the complete coding sequence of the HIV-1_{NL4-3} *vpr* gene to the yeast Gal4DB in a plasmid that directs the expression of a fusion protein (Gal4DBVpr) in yeast. The target plasmid was cotransformed into the yeast reporter strain HF7c with a yeast expression library that directs expression of fusion proteins between the Gal4AD and HeLa cDNA-encoded proteins. We identified multiple positive clones. Among positives obtained in the two-hybrid screen we identified uracil DNA glycosylase (UNG) previously reported by others as a Vpr-interacting protein (4). Sequence analysis of one of the other positive clones revealed that the cDNA encoded the carboxy-terminal fragment of a protein identical to one of the human homologs of the *S. cerevisiae* Rad23 protein, HHR23A (34). An alignment of the two human homologs of Rad23, HHR23A and HHR23B, and the *S. cerevisiae* Rad23 protein is shown in Fig. 1. The N terminus

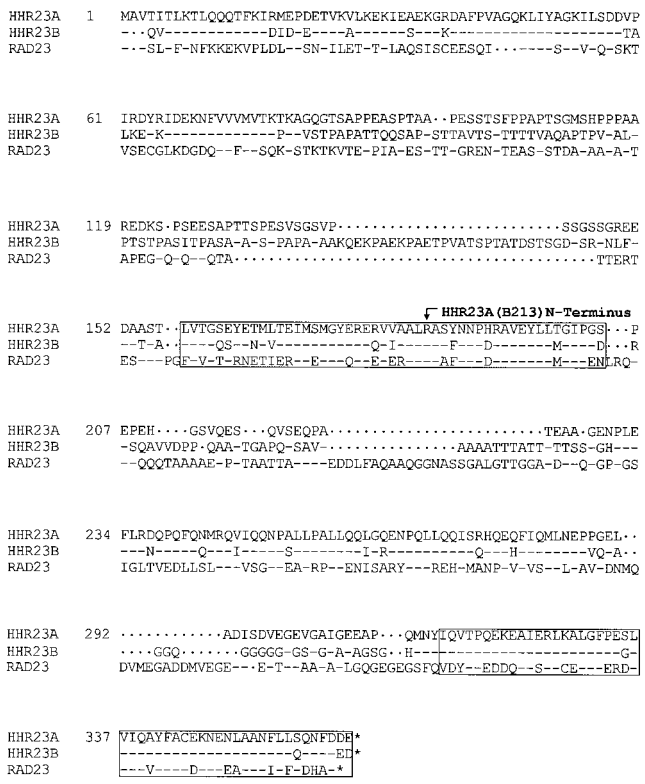

FIG. 1. Amino acid sequence alignment of the yeast *S. cerevisiae* Rad23 protein and the two human homologs HHR23A and HHR23B. The N terminus of the longest HHR23A clone isolated in the two-hybrid screen, HHR23A (B213), is indicated with an arrow above the HHR23A sequence. The highly conserved internal repeat domain homologous to members of the ubiquitin pathway is indicated by the boxed regions. The numbers on the left are those of the amino acid residues of HHR23A. (This figure is adapted from Fig. 1 of reference 47). The dots indicate gaps in the sequence, dashes indicate identical amino acids, and asterisks indicate stop codons.

of the longest cDNA clone we obtained in the two-hybrid screening, designated HHR23A(B213), is indicated above the HHR23A sequence. The yeast and human Rad23 proteins function in the nucleotide excision repair (NER) DNA repair pathway (35, 44). HHR23B functions in NER in the global genome repair pathway as part of a complex with the xeroderma pigmentosum complementation group C protein (XPC) (34). HHR23A has also been found to complex with XPC (17a). HHR23A and -B share extensive overall homology to each other and contain two copies of a highly conserved 50-aa acidic domain that is conserved among all the Rad23 homologs (see Fig. 1). This acidic repeat domain shares homology to the C-terminal extension of a bovine ubiquitin conjugating enzyme (UBC), E2(25K) (47). HHR23A(B213) encodes the C-terminal portion of HHR23A which includes the entire C-terminal conserved repeat domain and a portion of the internal conserved repeat domain.

It is noteworthy that the strain of yeast expressing the Gal4DBVpr fusion was growth impaired compared to those expressing the Gal4DB alone or a fusion between the Gal4DB and the yeast kinase SNF1 (Gal4DBSNF1). This suggested that expression of the Gal4DBVpr fusion induced growth arrest to some extent in *S. cerevisiae*, as has been reported by other investigators (33). The presence of the plasmid encoding Gal4ADHHR23A(B213) resulted in the restoration of a normal growth rate. Restoration of the growth-impaired phenotype was not seen when either Gal4AD alone or a fusion between the Gal4AD and an irrelevant protein (Gal4ADSNF4) was coexpressed with Gal4DBVpr (data not shown). These controls allowed us to establish that the Gal4DBVpr fusion protein (i) interacted specifically with Gal4ADHHR23A (B213) and (ii) interacted with the HHR23A portion of the Gal4ADHHR23A(B213) fusion protein and not the Gal4AD portion.

Interaction between GSTVpr and HHR23A expressed in cells. In order to confirm a direct physical interaction between HHR23A and Vpr, we tested the ability of the full-length HHR23A protein transiently expressed in HeLa cells to bind to a recombinant fusion protein between GST and Vpr (GSTVpr). The complete coding sequence of the HIV-1_{NL4-3} *vpr* gene was ligated to GST sequences in a plasmid that allowed expression of a chimeric fusion protein in bacteria. GSTVpr was purified from bacteria by affinity chromatography on glutathione-Sepharose. To produce full-length HHR23A we constructed an expression vector containing the complete coding sequence of HHR23A with an N-terminal FLAG epitope tag to facilitate detection by Western blotting. (These plasmids were also cotransfected with a plasmid encoding Vpr or a mutant form of Vpr, VprX, for subsequent cell cycle analysis [see below].) Forty-eight hours after transfection, HeLa cells were lysed and lysates were analyzed by Western blotting. Both the full-length HHR23A and HHR23A(B213) proteins were expressed in HeLa cells (Fig. 2A). Lysates were then used in an ex vivo binding assay. GSTVpr-associated proteins were selectively recovered by affinity binding to glutathione-Sepharose beads. The ability of HHR23A to bind to GSTVpr was determined by Western blot analysis of GSTVpr-associated proteins with a monoclonal antibody directed toward the M2 FLAG epitope (Fig. 2B). We detected HHR23A bound to GSTVpr but were unable to detect HHR23A when GST was used in the binding reaction. This result provides evidence for a direct interaction between Vpr and the full-length HHR23A and confirms and extends the genetic data obtained through the yeast two-hybrid screen.

Subcellular localization and colocalization of Vpr and HHR23A in transfected cells. Since we detected binding of the full-length HHR23A to GSTVpr, we determined whether these proteins colocalized to the same subcellular compartments and whether the subcellular localization of the individual proteins, when expressed alone, was altered by the presence of the other. Vpr has previously been reported to localize in the nucleus (9a). Similarly, indirect immunofluorescence studies of endogenous HHR23A in HeLa cells have demonstrated nuclear localization during interphase (46).

To define the intracellular distribution of Vpr and HHR23A we performed indirect immunofluorescence assays of HeLa cells expressing either Vpr, HHR23A, or the truncated form, HHR23A(B213), alone or Vpr and HHR23A or HHR23A (B213) together and analyzed them by confocal microscopy. We found that HHR23A is localized primarily in the perinuclear region (Fig. 3A). HHR23A(B213) was also localized primarily in the perinuclear region (Fig. 3D). The discrepancy in cellular localization of HHR23A with the previously observed nuclear localization may be due to differences in the fixation and staining protocols or to our use of confocal microscopy. As previously reported, Vpr localized primarily in the nucleus (Fig. 3B and E) (9a). We also observed a concentration of Vpr in the perinuclear region. In cells expressing both Vpr and HHR23A or HHR23A(B213), there was a colocalization of Vpr and HHR23A within the nucleus and, in particular, in the perinuclear region (Fig. 3C). Similar colocalization was observed with Vpr and HHR23A(B213) (Fig. 3F). As a further

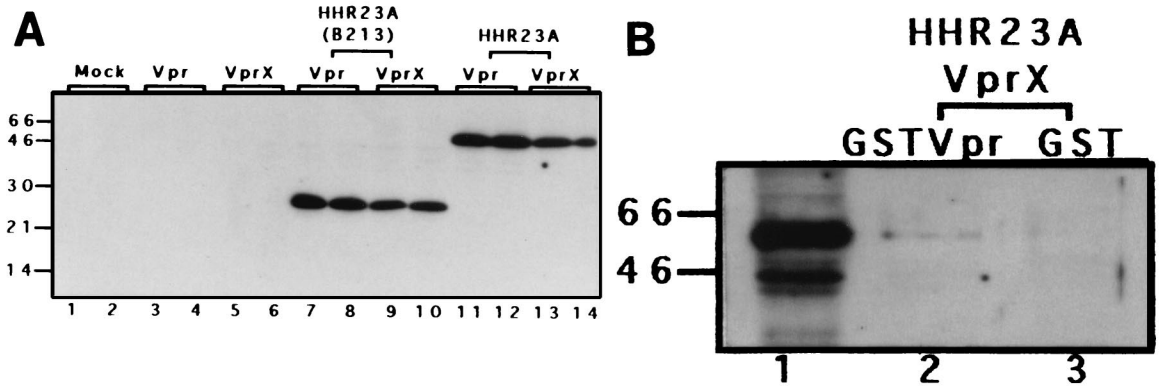

FIG. 2. (A) Expression of full-length HHR23A and HHR23A(B213) in HeLa cells. HeLa cells were transfected by using Lipofectin with a Vpr expression vector, BSVprThy, or a mutant Vpr expression vector, BSVprXThy, and a 20-fold molar excess of either pXC, the full-length HHR23A expression vector pXCR23A, or pXCB213, which expresses the longest HHR23A cDNA isolated in the two-hybrid screen. Forty-eight hours after transfection lysates were prepared and analyzed by Western blotting. Blots were sequentially incubated with mouse monoclonal antibodies specific for the M2 FLAG epitope and a horseradish peroxidase-linked sheep anti-mouse immunoglobulin. Antibody binding was detected by using the ECL detection kit. All transfections were performed in duplicate. The full-length M2-tagged HHR23A protein migrates between the 45- and 66-kDa molecular mass markers, and the M2-tagged HHR23A(B213) protein migrates just below the 30-kDa marker. (B) GSTVpr binds to full-length HHR23A expressed in cells. GSTVpr or GST was mixed with lysates isolated from HeLa cells co-transfected with BSVprXThy and pXCR23A (full-length HHR23A). After a 4-h incubation at 4°C with gentle rocking, GST-bound proteins were selectively recovered with the addition of glutathione-Sepharose. Protein complexes bound to glutathione beads were subjected to PAGE, transferred to nitrocellulose, and visualized by chemiluminescence after sequential binding of a primary monoclonal antibody specific for the M2 epitope and a secondary antibody conjugated to horseradish peroxidase. Lane 1, lysate (5 μl) from HeLa cells cotransfected with BSVprXThy and pXCR23A and used in the binding assay; lane 2, lysate from BSVprXThy-pXCR23A cotransfected cells (30 μl) incubated with GSTVpr; lane 3, lysate from BSVprXThy-pXCR23A cotransfected cells (30 μl) incubated with GST. The position of the full-length M2-tagged HHR23A is just below the 66-kDa molecular mass marker. The Western blot shown was also probed with an antibody to GST (data not shown). Equal amounts of GSTVpr and GST were recovered on the glutathione-Sepharose beads.

control, we tested whether another nuclear protein, Cdc2, would colocalize with HHR23A(B213). When the proteins were coexpressed in cells, Cdc2 was located primarily throughout the nucleus (Fig. 3H) and HHR23A(B213) was located in the perinuclear region (Fig. 3G). The proteins did not colocalize within the perinuclear region (Fig. 3I). These results demonstrate that Vpr and HHR23A, when coexpressed in cells, are both present within the same subcellular region. The colocalization of HHR23A and Vpr in cells is consistent with the demonstration of a physical interaction between HHR23A and GSTVpr.

Mapping domains of HHR23A which interact with Vpr. We screened the panel of positive clones identified in the two-hybrid screen to identify all Gal4ADHHR23A cDNA fusion proteins encoding fragments of the HHR23A coding sequences which interacted with Vpr. We identified a total of eight Gal4ADHHR23A clones encoding fusion proteins of various lengths which maintained interaction with Gal4DBVpr in the yeast two-hybrid screen. Sequence analysis of these clones identified a common 45-aa C-terminal region of HHR23A which was present in all clones (Fig. 4). Interestingly, this domain corresponds to the 50-aa domain identified by sequence analysis that shares homology to members of the ubiquitination pathway including the C-terminal extension of a bovine ubiquitin conjugating enzyme, E2(25K), which is thought to promote interaction with its substrate and/or function in cellular localization.

The 45-aa C-terminal portion of HHR23A is sufficient for binding to GSTVpr. To further characterize the HHR23A sequences required for interaction with Vpr we examined the ability of the 45-aa HHR23A domain identified in the two-hybrid system to bind to GSTVpr in vitro. We chemically synthesized a peptide corresponding to the 45-aa C-terminal portion of HHR23A and tested it for binding to the GSTVpr fusion protein (Fig. 5A). To visualize the peptide following affinity binding with glutathione-Sepharose and Western blotting, the peptide was synthesized with a biotin tag on the amino terminus. We found that the peptide derived from the 45-aa C terminus of HHR23A specifically bound to GSTVpr but not to GST and binding could be competed with increasing amounts of unlabeled HHR23A peptide (Fig. 5B). These results confirm that the conserved carboxy-terminal region of HHR23A comprises a binding domain for Vpr.

There are two homologs of the yeast Rad23 gene product in human cells, HHR23A, identified here, and HHR23B. The 45-aa C-terminal portion of HHR23A constitutes most of the 50-aa repeat element that is highly conserved between the two human homologs: the corresponding region of HHR23B differs by only 4 aa (see Fig. 1). Thus, we determined if a synthetic peptide derived from the 45-aa C-terminal region of HHR23B would also bind to GSTVpr in the in vitro assay. We found that the HHR23B peptide did specifically bind to GSTVpr but not to GST (Fig. 5A). Thus, although not detected through the yeast two-hybrid screen, it is possible that HHR23B may also interact with Vpr in cells.

Overexpression of HHR23A partially alleviates Vpr-mediated cell cycle arrest. Expression of Vpr in cells results in an arrest at the G_2 phase of the cell cycle. One prediction for a cellular protein that interacts with Vpr and that is involved in the cell cycle arrest phenotype would be that overexpression might result in alleviation of Vpr-induced G_2 arrest. We tested this hypothesis by cotransfecting HeLa cells with HHR23A (B213) and Vpr expression plasmids. The HHR23A(B213) expression plasmid pXCB213 encodes the above-mentioned C-terminal portion of HHR23A which includes the entire C-terminal conserved repeat domain and a portion of the internal conserved repeat domain. The Vpr expression plasmid BSVprThy contains, in addition to the Vpr coding sequences, the Thy1.2 cell surface reporter which allows us to distinguish by flow cytometry successfully transfected cells expressing Thy1.2 and to simultaneously assess cell cycle status of transfected and nontransfected cells (23). The HHR23A(B213)-encoding construct was cotransfected in approximately a 20-fold molar excess over BSVprThy to ensure that the majority of cells which

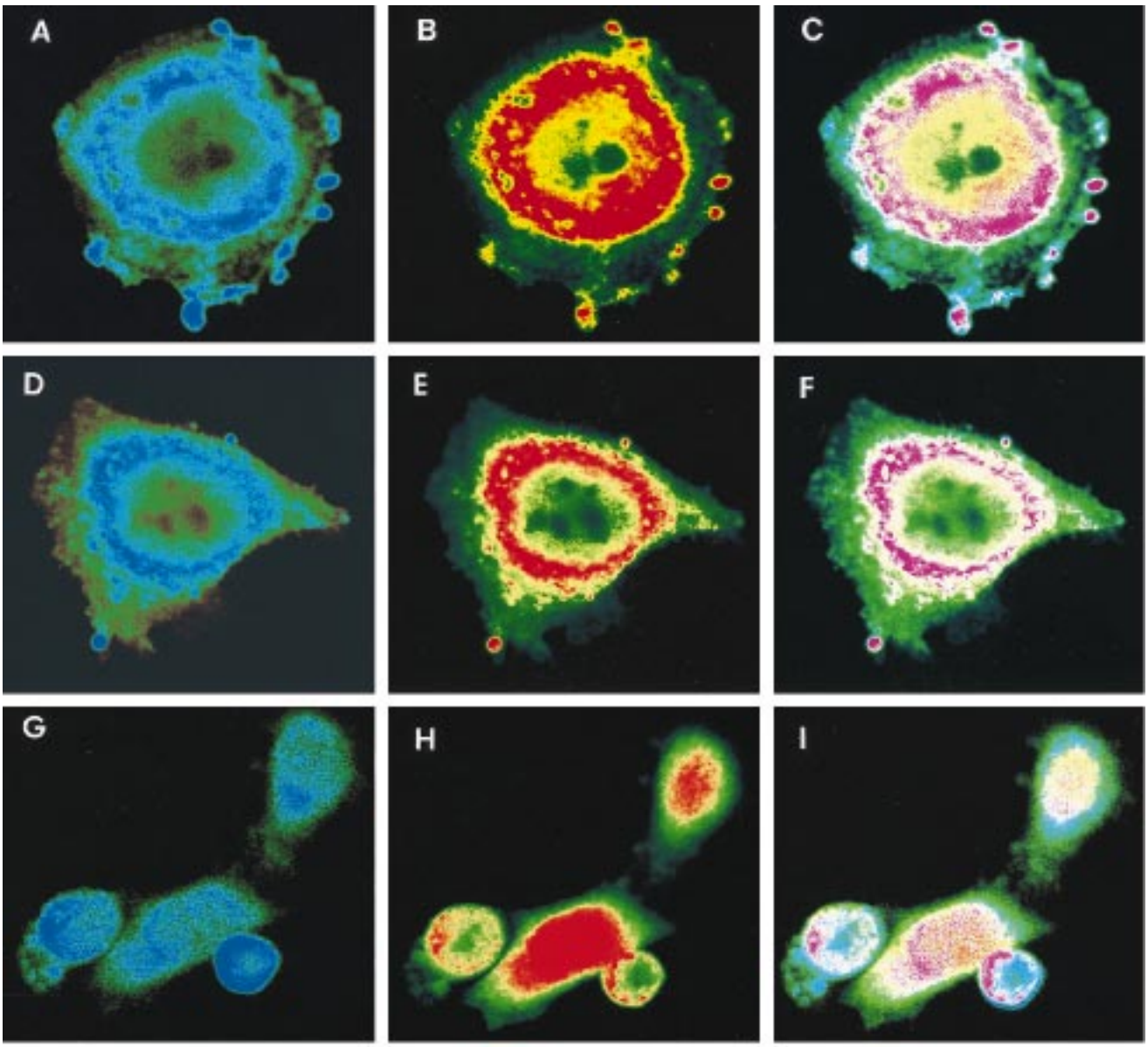

FIG. 3. HHR23A colocalizes with Vpr in transfected HeLa cells. HeLa cells were transfected with the indicated expression plasmids as described in Materials and Methods. Cells were stained with 12 CA5 monoclonal antibody (Vpr, Cdc2) or M2 FLAG monoclonal antibody [HHR23A, HHR23A(B213)] as described in Materials and Methods. Images were acquired on a Zeiss confocal microscope, and the fluorescence intensity was coded into the red half (Vpr, Cdc2) or the blue half [HHR23A, HHR23A(B213)] of the spectrum. Images acquired at the same plane were superimposed to show colocalization, which appears as lavender. (A) HHR23A, blue; (B) Vpr, red; (C) HHR23A and Vpr, lavender; (D) HHR23A(B213), blue; (E) Vpr, red; (F) HHR23A(B213) and Vpr, lavender; (G) HHR23A(B213), blue; (H) Cdc2, red; (I) HHR23A(B213) and Cdc2, lavender.

express Vpr and Thy1.2 would also express HHR23A(B213). Figure 6a shows the effect of Vpr on transfected cells. The Thy1.2-negative cells (to the left of the *x* axis gate) show a normal cycling cell cycle profile. The Thy1.2-positive cells (to the right of the *x* axis gate) are transfected and show the typical arrested phenotype of cells expressing Vpr (23). These cells expressing Thy1.2 and Vpr showed a G_1/G_2 ratio of 0.15 (Fig. 6a, panel A). We found that coexpression of Vpr and HHR23A(B213) resulted in a significant reduction in the degree of cell cycle arrest. The G_1/G_2 ratio of the Thy1.2$^+$ population was approximately 1.2 (Fig. 6a, panel C). Cells that were mock transfected or transfected with a plasmid encoding a mutant form of Vpr, VprX, had a normal cell cycle profile with G_1/G_2 ratios of approximately 2.8 and 2.1, respectively (Fig. 6a, panels E and B). These results demonstrate that overexpression of a fragment of HHR23A which contains the region required for binding to GSTVpr in vitro results in a partial alleviation of Vpr-induced cell cycle arrest. This result provides support for the hypothesis that the interaction of Vpr with HHR23A is critical for the Vpr-induced cell cycle arrest phenotype.

We also tested whether coexpression of full-length HHR23A and Vpr would alleviate the Vpr-induced cell cycle arrest (Fig. 6b) (for ease of visualization, only the Thy1.2$^+$ populations are shown). Our results demonstrate that expression of full-length HHR23A alleviated Vpr-induced cell cycle arrest (Fig. 6b,

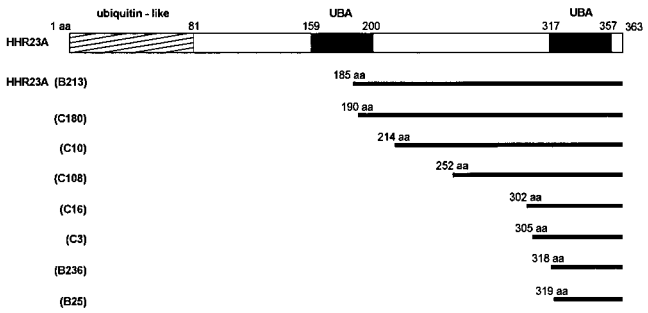

FIG. 4. Alignment of HHR23A and the eight different cDNA clones identified in the two-hybrid screen for proteins that interact with HIV-1 Vpr. The cDNAs encode proteins with the following lengths: B25, 45 aa; B236, 46 aa; C3, 59 aa; C16, 62 aa; C108, 112 aa; C10, 150 aa; C180, 174 aa; B213, 179 aa. The highly conserved internal repeat UBA domains are indicated as black boxes.

panel E) although consistently to a lesser extent than that observed with HHR23A(B213) (compare panels C and E).

We conducted further studies to demonstrate that the partial alleviation of Vpr-induced G_2 arrest is due to an intracellular interaction of Vpr with the C-terminal portion of HHR23A. An HHR23A mutant, HHR23A-trunc, that has a frameshift in the middle of the C-terminal Vpr binding domain at aa 346 was tested for its ability to alleviate Vpr-induced G_2 arrest. This frameshift results in expression of a protein that contains 10 additional aa following the frameshift site and deletion of the C-terminal 17 aa of HHR23A. We found that this mutation at the C terminus of HHR23A was sufficient to abolish the alleviation of G_2 arrest seen with the full-length wild-type protein (Fig. 6b, panel G). In addition, deletion of 45 aa of the highly conserved C-terminal UBA domain from HHR23A(B213) results in abrogation of alleviation of Vpr-induced G_2 arrest (data not shown). In order to rule out the possibility that alleviation of G_2 arrest was due to inhibition of Vpr expression when HHR23A or HHR23A(B213) were overexpressed in cells we assessed the relative levels of Vpr expression in the presence and absence of HHR23A expression. Western blots of cell lysates from the samples shown in Fig. 6b were probed simultaneously with antibodies to the hemagglutinin (Vpr) and M2 (HHR23A) epitope tags. We saw similar levels of Vpr expression in lysates regardless of the presence of HHR23A, HHR23A-trunc, or HHR23A(B213) (data not shown). These results provide further evidence for the interaction between the C-terminal portion of HHR23A and Vpr.

DISCUSSION

Our results demonstrate that Vpr binds directly to a human cellular protein, HHR23A. Using the yeast two-hybrid system to screen for cDNAs encoding cellular proteins that interact with HIV-1 Vpr, we have isolated a cDNA which partially encodes one of the human homologs of the yeast *rad23* gene, HHR23A. We have also demonstrated binding of full-length HHR23A from cell lysates with a recombinant GST-Vpr fusion protein. We confirmed the interaction between Vpr and HHR23A in vitro using recombinant proteins and synthetic peptides and mapped the Vpr interaction domain to a C-terminal domain of HHR23A. We demonstrated colocalization of HHR23A and Vpr in HeLa cells transiently expressing immunotagged HHR23A and Vpr by indirect immunofluorescence and confocal microscopy. Most significantly, we have shown that overexpression of HHR23A leads to a partial alleviation of Vpr-induced G_2 arrest. This finding provides functional evidence that Vpr and HHR23A interact in cells and that this interaction has biological consequences with regard to Vpr-mediated cell cycle arrest.

Cellular function of HHR23A. HHR23A is one of two human homologs of the *S. cerevisiae rad23* gene (34). The *rad23* gene is a nonessential gene that encodes a 42-kDa acidic protein that functions in NER in yeast (44). Ex vivo coimmunoprecipitation studies have demonstrated that yeast Rad23 is one component of a higher-order protein complex consisting of the multisubunit transcription factor TFIIH and Rad14, a zinc metalloprotein that binds specifically to UV-damaged

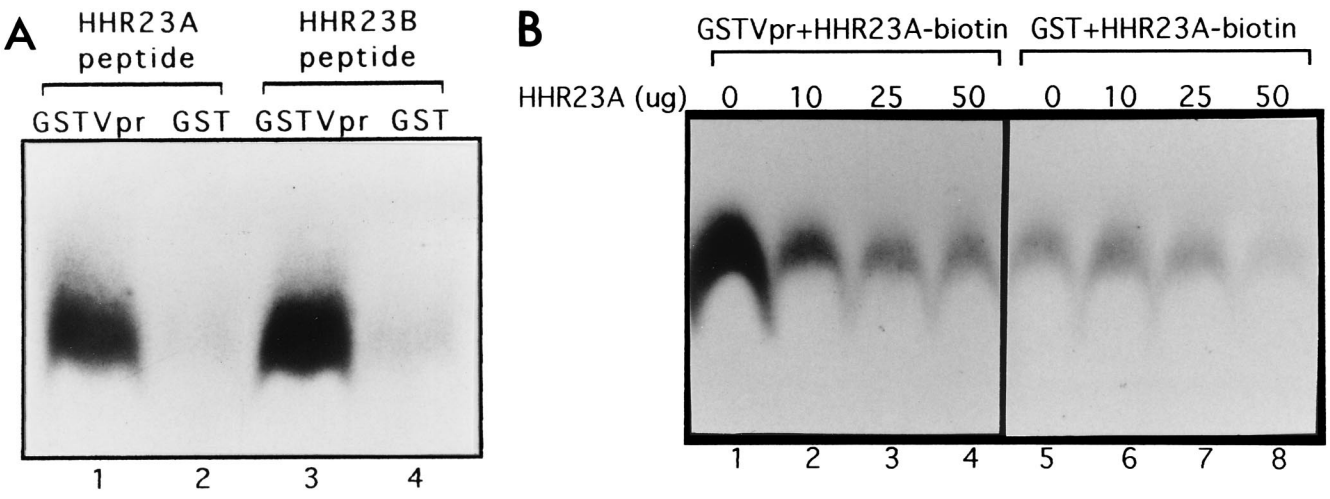

FIG. 5. GSTVpr binds to the 45-aa C-terminal portion of HHR23A and HHR23B which includes the highly conserved internal repeat domain. (A) Ten micrograms of GSTVpr or GST was mixed with 50 μg of biotinylated HHR23A or HHR23B peptide and incubated at 4°C for 1 h. GST-containing complexes were selectively recovered by using glutathione-Sepharose beads. Protein complexes bound to the glutathione beads were subjected to PAGE, transferred to nitrocellulose, and visualized with streptavidin conjugated to horseradish peroxidase by using chemiluminescence. (B) The 45-aa C terminus of HHR23A bound specifically to GSTVpr and could be competed with increasing amounts of unlabeled HHR23A peptide. Ten micrograms of GSTVpr or GST was mixed with 50 μg of biotinylated HHR23A or HHR23B peptide and incubated at 4°C for 1 h. Increasing amounts of unbiotinylated HHR23A peptide were then added to the binding reactions. GST-containing complexes were selectively recovered and visualized as described for panel A. The Western blots shown in panels A and B were also probed with an antibody to GST (data not shown). Equivalent amounts of GSTVpr and GST were recovered on the glutathione-Sepharose beads.

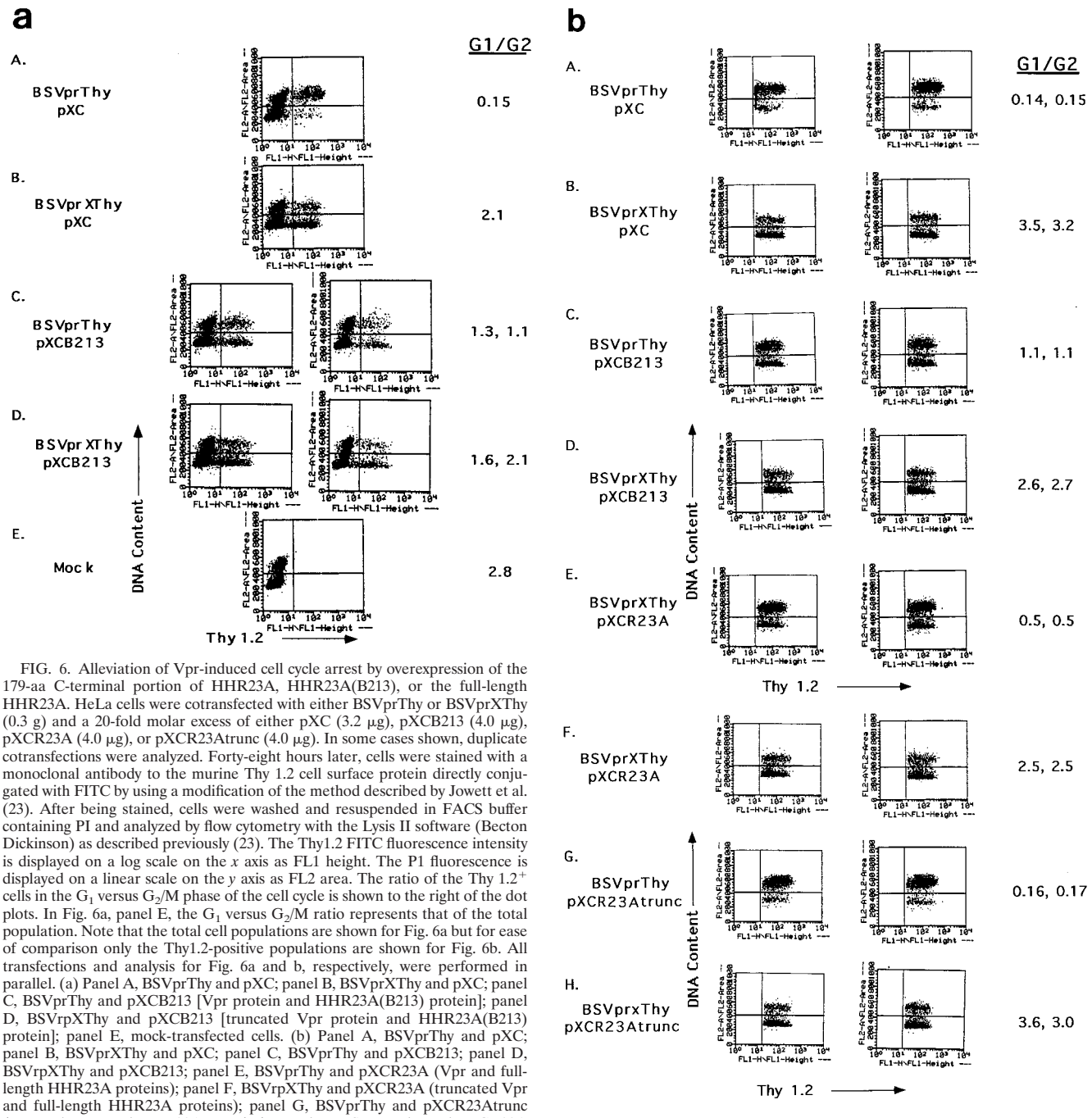

FIG. 6. Alleviation of Vpr-induced cell cycle arrest by overexpression of the 179-aa C-terminal portion of HHR23A, HHR23A(B213), or the full-length HHR23A. HeLa cells were cotransfected with either BSVprThy or BSVprXThy (0.3 g) and a 20-fold molar excess of either pXC (3.2 μg), pXCB213 (4.0 μg), pXCR23A (4.0 μg), or pXCR23Atrunc (4.0 μg). In some cases shown, duplicate cotransfections were analyzed. Forty-eight hours later, cells were stained with a monoclonal antibody to the murine Thy 1.2 cell surface protein directly conjugated with FITC by using a modification of the method described by Jowett et al. (23). After being stained, cells were washed and resuspended in FACS buffer containing PI and analyzed by flow cytometry with the Lysis II software (Becton Dickinson) as described previously (23). The Thy1.2 FITC fluorescence intensity is displayed on a log scale on the *x* axis as FL1 height. The P1 fluorescence is displayed on a linear scale on the *y* axis as FL2 area. The ratio of the Thy 1.2^+ cells in the G_1 versus G_2/M phase of the cell cycle is shown to the right of the dot plots. In Fig. 6a, panel E, the G_1 versus G_2/M ratio represents that of the total population. Note that the total cell populations are shown for Fig. 6a but for ease of comparison only the Thy1.2-positive populations are shown for Fig. 6b. All transfections and analysis for Fig. 6a and b, respectively, were performed in parallel. (a) Panel A, BSVprThy and pXC; panel B, BSVprXThy and pXC; panel C, BSVprThy and pXCB213 [Vpr protein and HHR23A(B213) protein]; panel D, BSVrpXThy and pXCB213 [truncated Vpr protein and HHR23A(B213) protein]; panel E, mock-transfected cells. (b) Panel A, BSVprThy and pXC; panel B, BSVprXThy and pXC; panel C, BSVprThy and pXCB213; panel D, BSVrpXThy and pXCB213; panel E, BSVprThy and pXCR23A (Vpr and full-length HHR23A proteins); panel F, BSVrpXThy and pXCR23A (truncated Vpr and full-length HHR23A proteins); panel G, BSVprThy and pXCR23Atrunc (Vpr and truncated HHR23A proteins); panel H, BSVrpXThy and pXCR23A trunc (truncated Vpr and truncated HHR23A proteins).

DNA (16, 17). Rad23 facilitates complex formation between Rad14 and TFIIH via interaction with Rad14 and the Rad25 and TFB1 components of TFIIH.

The cellular function of HHR23A and HHR23B is less well characterized than that of the yeast homolog. HHR23B was originally identified in association with the XPC protein, the putative homolog of the yeast Rad4 protein. XPC and HHR23B form a protein complex that corrects the DNA repair defects of human cells from patients with xeroderma pigmentosum complementation group C (XPC) (34). In vitro reconstitution studies have demonstrated that HHR23B exhibits a stimulatory effect on the correcting activity of XPC (44). These results indicate that HHR23B functions in the NER pathway known as the global genome repair pathway, the mode of repair that is defective in XPC cells. Recently, it has been shown that like HHR23B, HHR23A also binds to XPC both in vitro and in cells (17a, 30a). HHR23A also exhibits a stimulatory effect on the DNA repair correcting activity of XPC in vitro (17a). Unlike XPC, which exhibits a high affinity for single-stranded DNA, neither HHR23A or -B exhibits an affinity for single-stranded or double-stranded DNA.

Identification of the Vpr-interacting domain of HHR23A. HHR23A and its counterpart HHR23B encode acidic proteins of 40 and 43 kDa, respectively, that share extensive overall homology with each other (57% identity and 76% similarity) and with the *rad23* gene of *S. cerevisiae* (30 to 34% identity and 41% similarity). All of the Rad23 homologs identified to date, including human and mouse, share the following structure: a basic N-terminal ubiquitin-like domain followed by a highly conserved 50-aa acidic domain, termed the UBA domain, that is repeated at the C terminus. The function of the N-terminal ubiquitin-like domain is unknown. In *S. cerevisiae*, it is essential for the biological function of Rad23 but does not appear to mediate proteolytic degradation. The 50-aa repeated UBA domain shares homology with members of the ubiquitination pathway including a C-terminal extension of a bovine ubiquitin conjugating enzyme, E2(25K). Both repeats are fully conserved between the human and murine homologs, suggesting a functional role for this region possibly in the ubiquitination pathway (22, 47). The eight different HHR23A cDNAs isolated in the two-hybrid screen enabled us to localize the region of HHR23A required for interaction with Vpr to the C-terminal 45 aa of HHR23A. Peptides comprising a region as small as 45 aa are sufficient to bind to GST-Vpr. The corresponding 45 aa of HHR23B also bind to GSTVpr.

Models for Vpr-induced G_2 arrest. We previously proposed a hypothesis that Vpr may work by interfering with normal signal transduction processes involved in DNA repair (23, 40). Vpr may interact with a component(s) of a signal transduction pathway that (i) allows a cell to recognize the presence of DNA damage and thus arrest at the G_2 checkpoint, (ii) participates in DNA repair, or (iii) signals a cell that DNA damage is repaired and that mitosis can proceed. This proposal was based upon similarities between the effects of Vpr and the effects of certain DNA-damaging agents. Both Vpr and DNA-alkylating agents can cause cells to arrest or accumulate at the G_2 phase. Cdc2 kinase, a key regulator of the transition of cells from G_2 to M, is inactive in Vpr-arrested cells and in cells in which DNA is damaged by agents such as HN_2 (36, 37). Activation of Cdc2 kinase through dephosphorylation is necessary for the progression of cells into mitosis. In addition, a regulator of Cdc2 kinase, Cdc25, is also inactive in cells arrested by Vpr and DNA-damaging agents (42). We have recently shown that agents such as the methylxanthines, which can reverse the cell cycle arresting effects of DNA-alkylating agents and augment their cytotoxic effects, will also reverse the Vpr-induced G_2 arrest of cells. The results reported here provide additional support for the hypothesis discussed above in that we have now identified binding between Vpr and a specific cellular protein which is involved in NER of damaged DNA. In addition, these results now provide a potential biochemical mechanism for Vpr-mediated arrest and will allow experimental approaches to directly test our hypothesis.

Several potential mechanisms involving HHR23A can be considered for cell cycle arrest by Vpr. First, HHR23A may be involved in recognition of damaged DNA. In this case Vpr may activate HHR23A or the complex of which it is a part in a manner similar to that of altered bases, phosphodiester backbone, or adducts in damaged DNA. These signals, in turn, would activate the G_2 checkpoint and lead to the arrest of cells. The second possibility is that HHR23A plays either a direct role in DNA repair or acts indirectly as a component of a multiprotein DNA repair complex. Vpr binding to HHR23A would either inhibit the repair activity of HHR23A or prevent association of HHR23A with a complex and thereby prevent the formation of an appropriate active complex for NER. Thus, repair would not occur and pathways which signal a cell to progress normally into mitosis would be blocked, leading to the arrest at the G_2 checkpoint. A third possibility is that HHR23A is part of a cell pathway that signals when damaged DNA has been repaired and transit from G_2 to M should proceed. In this case binding of Vpr to HHR23A would interfere with the transmission of a signal by HHR23A, resulting in G_2 arrest.

Based upon the homology between HHR23A and the C-terminal extension of the bovine E2(25K) protein, one could also speculate about a possible effect of Vpr interaction with HHR23A. The E2(25K) protein is a class 2 ubiquitin conjugating enzyme (UBC). Class 2 UBCs contain a highly conserved catalytic domain followed by unrelated C-terminal extensions that vary in length and which are thought to promote interaction with their substrate and/or function in their cellular localization. The other class of UBCs, class 1 enzymes, lack C-terminal extensions and require auxiliary proteins (E3 proteins) for substrate recognition. Thus, HHR23A may act as an E3 protein and promote interaction with a substrate that is targeted for destruction by the ubiquitin pathway. Degradation of this substrate would be required for entry into mitosis. In this scenario Vpr binding to HHR23A would interfere with ubiquitination of a protein whose degradation is required for transition into M, resulting in G_2 arrest.

One prediction of our hypothesis that Vpr interaction with HHR23A is critical for cell cycle arrest is that overexpression of HHR23A would lead to alleviation of cell cycle arrest. Both full-length HHR23A and a truncated version that maintains the Vpr-binding region alleviated cell cycle arrest. The effect of full-length HHR23A on alleviating Vpr-induced cell cycle arrest is less than that seen with HHR23A(B213). The difference in the extent of alleviation of G_2 arrest between HHR23A (B213) and HHR23A may reflect differences in the binding affinities of the two proteins to Vpr. Another explanation is that there is a higher level of expression of the smaller protein, HHR23A(B213) (see Fig. 2). Alternatively, the differences may relate to other functional properties of HHR23A which would be absent in the truncated mutant protein.

Since the role of the HHR23A homolog in cells is not well defined it is also possible that the Vrp-HHR23A interaction may not be involved in the cell cycle arrest phenotype induced by Vpr but may be involved in mediating other Vpr functions such as nuclear import.

Relevance of the Vpr-HHR23A interaction in HIV-1 pathogenesis. The role for Vpr in HIV-1 pathogenesis is as yet unclear. We and others (19, 23) have proposed that the expression of Vpr enables HIV-1 to (i) maximize the output of virus, (ii) prevent or decrease the rate of apoptosis thereby increasing the amount of virus produced, (iii) increase resistance to cytotoxic T-cell attack by blocking activation of cdc2 kinase and preventing cells from cycling, and (iv) prevent clonal expansion of $CD4^+$ effector cells which recognize HIV-1. In this latter scenario Vpr would cripple the immune response against HIV-1 and thus facilitate persistence of the virus within the infected individual. In addition the effect of Vpr on the general immune response may play a role in the development of AIDS. The identification of HHR23A as a Vpr-interacting protein and the demonstration that overexpression of HHR23A can alleviate cell cycle arrest may provide a basis for the future development of a new class of anti HIV-1 therapeutic agents. A better understanding of the function of HHR23A and its interaction with Vpr should provide important information on how these proteins may regulate and perturb cell growth, respectively.

ACKNOWLEDGMENTS

The work was supported in part by NIH grant CA-70018 and Amgen. E.S.W.-W. was supported by a Tumor Immunology Institutional Training Grant (CA09120).

The HeLa cDNA library, *E. coli* MH4, and the yeast strain HF7c used in the two-hybrid screening were generously supplied by Gregory Hannon and David Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). pAS2 and the Gal4DBSNF1 and Gal4ADSNF4 fusion plasmids were generously supplied by Stephen Elledge (University of Texas at San Antonio). We thank Marco A. Vasquez and Keith Parker for help with confocal image processing and microscopy, respectively, Laarni Antonio and Dave Grosshans for nucleotide sequence analysis, and Ling Lin for manuscript preparation.

REFERENCES

1. **Agostini, I., J. M. Navarro, F. Rey, M. Bouhamdan, B. Spire, R. Vigne, and J. Sire.** 1996. The human immunodeficiency virus type 1 Vpr transactivator: cooperation with promoter-bound activator domains and binding to TFIIB. J. Mol. Biol. **261:**599–606.

1a. **Ausubel, F. M.** 1988. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.

2. **Balliet, J. W., D. L. Kolson, G. Eiger, F. M. Kim, K. A. McGann, A. Srinivasan, and R. Collman.** 1994. Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes *vpr*, *vpu*, and *nef*. mutational analysis of a primary HIV-1 isolates. Virology **200:**623–631.

3. **Bartz, S. R., M. E. Rogel, and M. Emerman.** 1996. Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G_2 accumulation by a mechanism which differs from DNA damage checkpoint control. J. Virol. **70:**2324–2331.

4. **Bouhamdan, M., S. Benichou, F. Rey, J. M. Navarro, I. Agostini, B. Spire, J. Camonis, G. Slupphagu, R. Vigne, R. Bernarous, and J. Sire.** 1996. Human immunodeficiency virus type 1 Vpr protein binds to the uracil DNA glycosylase DNA repair enzyme. J. Virol. **70:**697–704.

5. **Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubei, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson.** 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature **365:**666–669.

6. **Bukrinsky, M. I., N. Sharova, T. L. McDonald, T. Pushkarskaya, W. G. Tarpley, and M. Stevenson.** 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc. Natl. Acad. Sci. USA **90:**6125–6129.

7. **Cohen, E. A., G. Dehni, J. G. Sodroski, and W. A. Haseltine.** 1990. Human immunodeficiency virus *vpr* product is a virion-associated regulatory protein. J. Virol. **64:**3097–3099.

8. **Cohen, E. A., E. F. Terwiliger, Y. Jalinoos, J. Proulx, J. G. Sodroski, and W. A. Haseltine.** 1990. Identification of HIV-1 *vpr* product and function. J. Acquired Immune Defic. Syndr. **3:**11–18.

9. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type 1 in mononuclear phagocytes. Virology **206:**935–944.

9a. **Di Marzio, P., S. Choe, M. Ebright, R. Knoblauch, and N. R. Landau.** 1995. Mutational analysis of cell cycle arrest, nuclear localization, and virion packaging of human immunodeficiency virus type 1 Vpr. J. Virol. **69:**7909–7916.

10. **Feilotter, H. E., G. J. Hannon, C. J. Ruddell, and D. Beach.** 1994. Construction of an improved host strain for two hybrid screening. Nucleic Acids Res. **22:**1502–1503.

11. **Fields, S., and O. K. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245–246.

12. **Fletcher, T. M., III, B. Brichacek, N. Sharova, M. A. Newman, G. Stivahtis, P. M. Sharp, M. Emerman, B. H. Hahn, and M. Stevenson.** 1996. Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV_{SM}. EMBO J. **15:**6155–6165.

13. **Freed, E. O., G. Englund, and M. A. Martin.** 1995. Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection. J. Virol. **69:**3949–3954.

14. **Gibbs, J. S., A. A. Lackner, S. M. Lang, M. A. Simon, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1995. Progression to AIDS in the absence of a gene for *vpr* or *vpx*. J. Virol. **69:**2378–2383.

15. **Guan, K., and J. E. Dixon.** 1991. Eukaryotic proteins expressed in E. coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. **192:**262–267.

16. **Guzder, S. N., V. Bailly, P. Sung, L. Prakash, and S. Prakash.** 1995. Yeast DNA repair protein Rad23 promotes complex formation between transcription factor TFIIH and DNA damage recognition factor Rad14. J. Biol. Chem. **270:**8385–8388.

17. **Guzder, S. N., Y. Habraken, P. Sung, L. Prakash, and S. Prakash.** 1995. Reconstitution of yeast nucleotide excision repair with purified Rad proteins, replication protein A, and transcription factor TFIIH. J. Biol. Chem. **270:** 12973–12976.

17a. **Hanaoka, F. (Osaka University).** Personal communication.

18. **Hannon, G. J., D. Demetrick, and D. Beach.** 1993. Isolation of the RB-related p130 through its interaction with Cdk2 and cyclins. Genes Dev. **7:**2378–2391.

19. **He, J. L., S. Choe, R. Walker, P. D. Marzio, D. O. Morgan, and N. R. Landau.** 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G_2 phase of the cell cycle by inhibiting $p34^{cdc2}$ activity. J. Virol. **69:**6705–6711.

20. **Heinzinger, N. K., M. I. Bukrinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA **91:**7311–7315.

21. **Hoch, J., S. M. Lang, M. Weeger, C. Stahl-Hennig, C. Coulibaly, U. Dittmer, G. Hunsmann, D. Fuchs, J. Muller, S. Sopper, B. Fleckenstein, and K. T. Uberla.** 1995. *vpr* deletion mutant of simian immunodeficiency virus induces AIDS in rhesus monkeys. J. Virol. **69:**4807–4813.

22. **Hofmann, K., and P. Bucher.** 1996. The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. Trends Biochem. Sci. **21:**172–173.

23. **Jowett, J. B. M., V. Planelles, B. Poon, N. P. Shah, M. L. Chen, and I. S. Y. Chen.** 1995. The human immunodeficiency virus type 1 *vpr* gene arrests infected T cells in the G_2 + M phase of the cell cycle. J. Virol. **69:**6304–6313.

24. **Kondo, E., F. Mammano, E. A. Cohen, and H. G. Göttlinger.** 1995. The $p6^{gag}$ domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. J. Virol. **69:**2759–2764.

25. **Krishan, A.** 1975. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J. Cell Biol. **66:**188–193.

26. **Lang, S. M., M. Weeger, S. H. Christiane, C. Coulibaly, G. Hunsmann, J. Muller, H. Muller-Hermelink, D. Fuchs, H. Wachter, M. M. Daniel, R. C. Desrosiers, and B. Fleckenstein.** 1993. Importance of *vpr* for infection of rhesus monkeys with simian immunodeficiency virus. J. Virol. **67:**902–912.

27. **Lavallee, C., X. J. Yao, A. Ladha, H. Gottlinger, W. A. Haseltine, and E. A. Cohen.** 1994. Requirement of the $Pr55^{gag}$ precursor for incorporation of the Vpr product into human immunodeficiency virus type 1 viral particles. J. Virol. **68:**1926–1934.

28. **Lee, M. G., and P. Nurse.** 1987. Complementation used to clone a human homologue of the fission yeast cell cycle control gene *Cdc2*. Nature **327:**31–35.

29. **Levy, D. N., L. S. Fernandes, W. V. Williams, and D. B. Weiner.** 1993. Induction of cell differentiation by human immunodeficiency virus 1 *vpr*. Cell **72:**541–550.

30. **Levy, D. N., Y. Refaeli, R. R. MacGregor, and D. B. Weiner.** 1994. Serum Vpr regulates productive infection and latency of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **91:**10873–10877.

30a. **Li, L., X. Lu, C. Peterson, and R. Legerski.** 1997. XPC interacts with both HHR23B and HHR23A in vivo. Mut. Res. **383:**197–203.

31. **Lu, Y. L., R. P. Bennett, J. W. Wills, R. Gorelick, and L. Ratner.** 1995. A leucine triplet repeat sequence $(LXX)_4$ in $p6^{gag}$ is important for Vpr incorporation into human immunodeficiency virus type 1 particles. J. Virol. **69:** 6873–6879.

32. **Lu, Y. L., P. Spearman, and L. Ratner.** 1993. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. J. Virol. **67:**6542–6550.

33. **Macreadie, I. G., L. A. Castelli, D. R. Hewish, A. Kirkpatrick, A. C. Ward, and A. A. Azad.** 1995. A domain of human immunodeficiency virus type 1 Vpr containing repeated H(S/F)RIG amino acid motifs causes cell growth arrest and structural defects. Proc. Natl. Acad. Sci. USA **92:**2770–2774.

34. **Masutani, C., K. Sugasawa, J. Yanagisawa, T. Sonoyama, M. Ui, T. Enomoto, K. Takio, K. Tanaka, P. J. van der Spek, D. Bootsma, et al.** 1994. Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homologue of yeast RAD23. EMBO J. **13:**1831–1843.

35. **Mueller, J. P., and M. J. Smerdon.** 1996. Rad23 is required for transcription-coupled repair and efficient overall repair in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**2361–2368.

36. **O'Connor, P. M., D. K. Ferris, G. A. White, J. Pines, T. Hunter, D. L. Longo, and K. W. Kohn.** 1992. Relationships between Cdc2 kinase, DNA cross-linking, and cell cycle perturbations induced by nitrogen mustard. Cell Growth Differ. **3:**43–52.

37. **O'Connor, P. M., D. K. Ferris, M. Pagano, G. Draetta, J. Pines, T. Hunter, D. L. Longo, and K. W. Kohn.** 1993. G_2 delay induced by nitrogen mustard in human cells affects cyclin A/Cdk2 and cyclin B1/Cdc2-kinase complexes differently. J. Biol. Chem. **268:**8298–8308.

38. **Paxton, W., R. I. Connor, and N. R. Landau.** 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of *gag* and mutational analysis. J. Virol. **67:**7229–7237.

39. **Planelles, V., J. B. M. Jowett, Q. X. Li, Y. Xie, B. Hahn, and I. S. Y. Chen.** 1996. Vpr-induced cell cycle arrest is conserved among primate lentiviruses. J. Virol. **70:**2516–2524.

40. **Poon, B., J. B. M. Jowett, S. A. Stewart, R. W. Armstrong, G. M. Rishton, and I. S. Y. Chen.** 1997. Human immunodeficiency virus type 1 *vpr* gene induces similar phenotypic effects as the DNA alkylating agent, nitrogen mustard. J. Virol. **71:**3961–3971.
41. **Rafaeli, Y., D. N. Levy, and D. B. Weiner.** 1995. The glucocorticoid receptor type II complex is a target of the HIV-1 *vpr* gene product. Proc. Natl. Acad. Sci. USA **92:**3621–3625.
42. **Re, F., D. Braaten, E. K. Franke, and J. Luban.** 1995. Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G_2 by inhibiting the activation of $p34^{cdc2}$-cyclin B. J. Virol. **69:**6859–6864.
43. **Rogel, M. E., L. I. Wu, and M. Emerman.** 1995. The human immunodeficiency virus type 1 *vpr* gene prevents cell proliferation during chronic infection. J. Virol. **69:**882–888.
44. **Sugasawa, K., C. Masutani, A. Uchida, T. Maekawa, P. J. van der Spek, D. Bootsma, J. H. J. Hoeijmakers, and F. Hanaoka.** 1996. HHR23B, a human Rad23 homolog, stimulates XPC protein in nucleotide excision repair in vitro. Mol. Cell. Biol. **16:**4852–4861.
45. **Tristam, H. C., A. Marshall, A. Karpas, and F. Hill.** 1992. Evolution of the primate lentiviruses: evidence from *vpx* and *vpr*. EMBO J. **11:**3405–3412.
46. **van der Spek, P. J., A. Eker, S. Rademakers, C. Visser, K. Sugasawa, C. Masutani, F. Hanaoka, D. Bootsma, and J. H. J. Hoeijmakers.** 1996. XPC and human homologs of RAD23: intracellular localization and relationship to other nucleotide excision repair complexes. Nucleic Acids Res. **24:**2551–2559.
47. **van der Spek, P. J., C. E. Visser, F. Hanaoka, B. Smit, A. Hagemeijer, D. Bootsma, and J. H. J. Hoeijmakers.** 1996. Cloning, comparative mapping, and RNA expression of the mouse homologues of the *Saccharomyces cerevisiae* nucleotide excision repair gene *Rad23*. Genomics **31:**20–27.
48. **Wang, L., S. Mukherjee, F. Jia, O. Narayan, and L. J. Zhao.** 1995. Interaction of virion protein Vpr of human immunodeficiency virus type 1 with cellular transcription factor Sp1 and trans-activation of viral long terminal repeat. J. Biol. Chem. **270:**25564–25569.
49. **Yu, X., Z. Matsuda, Q. C. Yu, T. H. Lee, and M. Essex.** 1990. Vpx of simian immunodeficiency virus is localized primarily outside the virus core in mature virions. J. Virol. **67:**4386–4390.
50. **Yuan, X., Z. Matsuda, M. Matsuda, M. Essex, and T. H. Lee.** 1990. Human immunodeficiency virus *vpr* gene encodes a virion-associated protein. AIDS Res. Hum. Retroviruses **6:**1265–1271.
51. **Zhao, L. J., S. Mukherjee, and O. Narayan.** 1994. Biochemical mechanism of HIV-I Vpr function—specific interaction with a cellular protein. J. Biol. Chem. **269:**15577–15582.
52. **Zhao, Y., J. Cao, M. R. G. O'Gorman, M. Yu, and R. Yogev.** 1996. Effect of human immunodeficiency virus type 1 protein R (*vpr*) gene expression on basic cellular function of fission yeast *Schizosaccharomyces pombe*. J. Virol. **70:**5821–5826.