

Anti-Vpr Activity of a Yeast Chaperone Protein

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Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) exerts multiple effects on viral and host cellular activities during viral infection, including nuclear transport of the proviral integration complex, induction of cell cycle G₂ arrest, and cell death. In this report, we show that a fission yeast chaperone protein Hsp16 inhibits HIV-1 by suppressing these Vpr activities. This protein was identified through three independent genome-wide screens for multicopy suppressors of each of the three Vpr activities. Consistent with the properties of a heat shock protein, heat shock-induced elevation or overproduction of Hsp16 suppressed Vpr activities through direct protein-protein interaction. Even though Hsp16 shows a stronger suppressive effect on Vpr in fission yeast than in mammalian cells, similar effects were also observed in human cells when fission yeast *hsp16* was expressed either in *vpr*-expressing cells or during HIV-1 infection, indicating a possible highly conserved Vpr suppressing activity. Furthermore, stable expression of *hsp16* prior to HIV-1 infection inhibits viral replication in a Vpr-dependent manner. Together, these data suggest that Hsp16 inhibits HIV-1 by suppressing Vpr-specific activities. This finding could potentially provide a new approach to studying the contribution of Vpr to viral pathogenesis and to reducing Vpr-mediated detrimental effects in HIV-infected patients.

Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr), a virion-associated protein of about 13 kDa, is highly conserved among HIV, simian immunodeficiency virus, and other lentiviruses (51, 52). Increasing evidence suggests that Vpr plays multiple roles during the viral life cycle including a possible role in promoting viral pathogenesis. For instance, Vpr displays several distinct activities in host cells. These include cytoplasmic-nuclear shuttling and nuclear localization to the nuclear membrane (25), induction of cell cycle G₂ arrest (24), and cell killing (48). The cell cycle G₂ arrest induced by Vpr is thought to suppress human immune functions by preventing T-cell clonal expansion (40) and to provide an optimized cellular environment for maximal levels of viral replication (22). The cytoplasmic-nuclear shuttling is believed to participate in nuclear transport of the viral preintegration complex (15, 25, 43). In addition, Vpr induces cell death. The biological significance of this effect is unclear at present, but it may contribute to the depletion of CD4⁺ T cells in HIV-infected patients (32, 41, 47). Consistent with the multiple roles Vpr plays during the viral life cycle, infections with Vpr-defective viruses in rhesus monkeys, chimpanzees, or human subjects are often associated with slower disease progression, and the mutant *vpr* often reverts back to the wild type (20, 22, 27, 47, 61). In vitro, Vpr is a moderate viral activator that contributes to a noticeable increase (two- to fourfold) in viral replication in some proliferating cells (22, 53, 59).

Early mutational analysis of the Vpr activities indicated that

three of the Vpr-specific activities such as localization to the nuclear membrane, induction of cell cycle G₂ arrest, and cell death are functionally independent of each other and have been demonstrated in a wide range of eukaryotic cells (9, 18). Consistent with this conservation, Vpr acts very similarly in fission yeast (*Schizosaccharomyces pombe*) and mammalian cells, making fission yeast a particularly useful model for studying Vpr activities (17, 62, 64). To further understand the potential roles of HIV-1 Vpr in the viral life cycle and to identify possible cellular factors antagonizing these Vpr effects, we used the fission yeast model system to launch three independent genome-wide searches for cellular proteins that are capable of suppressing each of these Vpr activities. Among the limited number of Vpr suppressors identified, a small heat shock protein (Hsp16) was found to specifically antagonize the Vpr activities of nuclear membrane localization, induction of cell cycle G₂ arrest, and cell death.

In this report, we describe specific suppression of the Vpr-related activities by Hsp16 and the conservation of this suppression in human cells and in the context of viral infections. In addition, we demonstrate that Hsp16 reduces viral replication in proliferating host cells in a Vpr-dependent manner.

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MATERIALS AND METHODS

Cell growth and gene expression in fission yeast cells. Genotypes and sources of *S. pombe* strains and plasmids used in this study are summarized in Table 1. Gene induction under the control of the fission yeast *nmt1* promoter in liquid medium has been described previously (36, 60). Briefly, cells containing the

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TABLE 1. Fission yeast strains, human cell lines, plasmids, and HIV-1 viral stocks

| Strains, cell lines, plasmids, and viral stocks | Genotype and characteristics | Source or reference |
|---|---|---------------------|
| S. pombe strains | | |
| SP223 | Wild type, <i>h⁻</i> , <i>ade6-216</i> , <i>leu1-32</i> , <i>ura4-294</i> | ATCC |
| Q812 | Wild type, <i>h⁻</i> , <i>leu1-32</i> , <i>ura4-294</i> | 49 |
| Q1649 | Wild type, <i>h⁻</i> , <i>leu1-32</i> , <i>ura4-294</i> , <i>gfp-hsp16</i> | 49 |
| RE076 | SP223 with single copy of F34Ivpr integrated at <i>ura4</i> locus; used for measuring Vpr-induced cell cycle G ₂ delay | 19 |
| RE007 | SP223 with single copy of <i>vpr</i> integrated at <i>ura4</i> locus; used for determining Vpr-induced cell killing | This study |
| RE078 | SP223 with single copy of <i>gfp-vpr</i> integrated at <i>ura4</i> locus; used for determining nuclear localization of Vpr | This study |
| Mammalian cell lines | | |
| HeLa | A cervical epithelial cell line | ATCC |
| H9 | CD4 ⁺ cells derived from PBMC for high-yield permissive growth with HIV-1 | 44 |
| CEM-SS | CD4 ⁺ cells derived from human T4-lymphoblastoid cells | 37 |
| 293T | An embryonic kidney fibroblast cell line | ATCC |
| 293T-632 | A 293T cell line expressing pVgR _{xR} and pCMV-EBNA1 | 66 |
| Plasmids | | |
| pYZIN | Fission yeast expression vector with an inducible <i>nmt1</i> promoter and a <i>leu1</i> selectable marker | 63 |
| pYZ3N | Same as pYZIN but with a 5' GFP-tag | 63 |
| pSF173 | Fission yeast expression vector with an inducible <i>nmt1</i> promoter and a 5' HA-tag, <i>ura4</i> selectable | ATCC |
| pZY1 | Mammalian expression vector with a muristerone A-inducible promoter; geneticin-resistant | 66 |
| pZH1 | Mammalian expression vector with a muristerone A-inducible promoter; hygromycin-resistant | 66 |
| pCDNA3.1 | Mammalian expression vector with a CMV promoter; hygromycin-resistant | Invitrogen |
| HIV-1 viral stocks | | |
| HIV-1 _{LAI} Vpr (+) | HIV-1 viral laboratory (subtype B) strain LAI | 4 |
| HIV-1 _{NL4-3} Vpr (+) | Vpr-positive NL4-3-based HIV-1 vector pNLHX | 8 |
| HIV-1 _{NL4-3} Vpr (-) | Same as above; <i>vpr</i> gene is mutated at the start codon from ATG to GTG | 43 |
| HIV-1 (VSV-G) Vpr (+) | Vpr-positive NL4-3-based HIV-1 vector. HIV-1 <i>env</i> gene is replaced by VSV-G gene | 8 |
| HIV-1 (VSV-G) Vpr (-) | Same as above; <i>vpr</i> gene is mutated at the start codon from ATG to GTG | 43 |

plasmid with the *nmt1* promoter were first grown to stationary phase in the presence of 20 μ M thiamine. Cells were then washed three times with distilled water, diluted to a final concentration of approximately 2×10^5 cells/ml in 10 ml of the appropriately supplemented Edinburgh minimal medium (EMM) with or without thiamine. Cells were examined 24 h after gene induction. All cells were normally grown at 30°C with constant shaking at 200 rpm unless otherwise specified.

Cell growth and gene expression in mammalian cells. Characteristics and sources of mammalian cell lines and plasmids used in this study are summarized in Table 1. CD4⁺ H9 and CEM-SS cells were grown in RPMI 1640 medium, and 293T cells were maintained in Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal calf serum and 100 U of penicillin-streptomycin per ml. The 293T-632 strain is a stable zeocin-resistant cell line (66) expressing a heterodimer of the modified ecdysone receptor (VgEcR) and the retinoid X receptor. The heterodimer binds a hybrid ecdysone response element (E/GRE) only in the presence of the synthetic analogue of ecdysone, muristerone A (38, 50). The transfected 293T-632 cells expressing *vpr* and Vpr suppressors were selected with Geneticin (750 μ g/ml; Mediatech) for *vpr* cloned in the pZY1 plasmid and hygromycin (200 μ g/ml) for genes cloned in the pZH1 plasmid (66). Gene induction from the muristerone A-inducible mammalian promoter has been described previously (66).

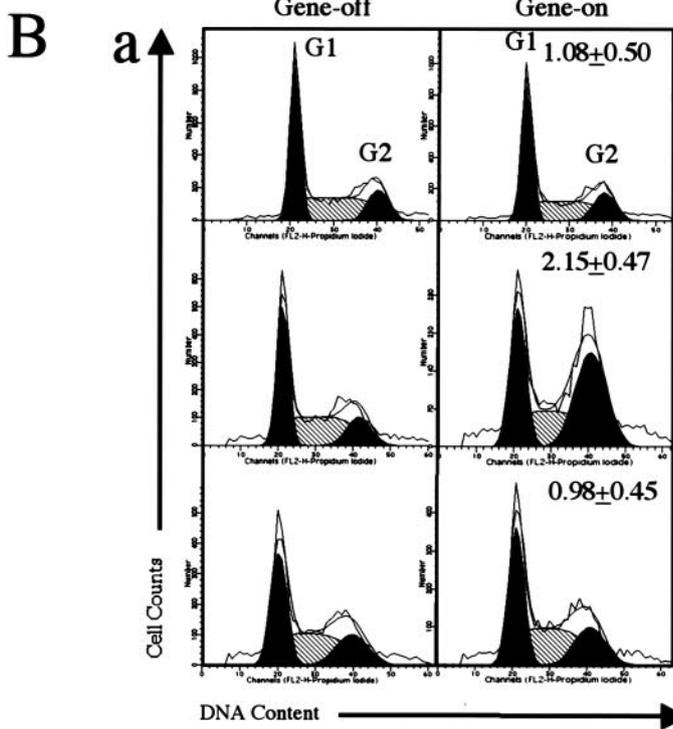
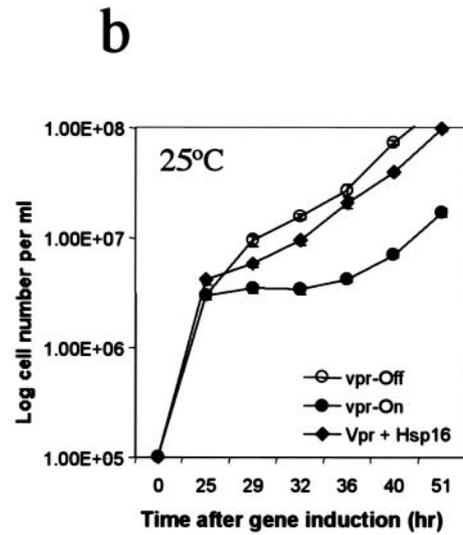
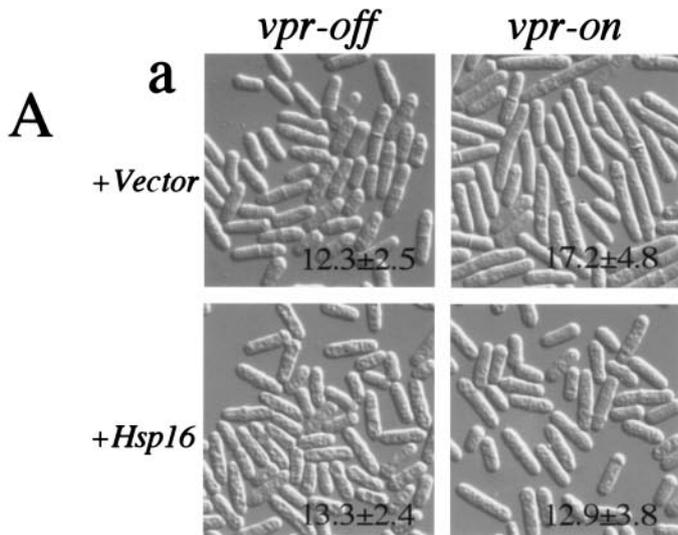
Viral infections. To test the effect of Hsp16 on Vpr-induced cell cycle G₂ arrest in the context of viral infection, we pseudotyped HIV-1 with a vesicular stomatitis virus G (VSV-G) envelope and infected 293T cells carrying the muristerone A-inducible *hsp16*-expressing vector. For pseudotyping, Vpr-positive and Vpr-negative matching infectious pNLHX clones (57) were cotransfected into 293T cells together with the VSV-G-expressing plasmid pHEF-VSVG obtained from the National Institutes of Health (NIH) AIDS Reagent Program (8). A total of 3×10^6 cells were infected with the VSV-G-pseudotyped Vpr-positive or Vpr-negative HIV-1 vector, and the cell cycle profile of infected cells was analyzed 48 h after infection. The Vpr-negative construct had a single base mutation at the

start codon of the *vpr* open reading frame, which changes ATG to GTG (43). The infected cells were normalized by reverse transcriptase (RT) activity to 3×10^6 cpm of RT activity per 10^6 cells.

To evaluate the potential suppressive effect of Hsp16 on cell killing caused by HIV infection, CD4⁺ H9 cells (44) that stably express a plasmid control and Hsp16 were established. A total of 3×10^6 to 5×10^6 of these H9 cells were either mock infected or infected with 2.0×10^3 times the 50% tissue culture infective dose of HIV-1_{LAI}. Equal infection of cells was further verified by measuring viral RNA levels 24 h after viral infection by using the Roche Monitor assay, according to the manufacturer's instructions. Prior to preparing the cells for RNA determination, the infected cells were washed three times with 20 \times phosphate-buffered saline (PBS) to remove the cell-free virus. These measurements showed that the viral RNA loads were 7.65×10^5 copies/ml for H9 without Hsp16 and 6.92×10^5 copies/ml for H9 cells with Hsp16. Since variation of this assay is normally within threefold, the viral RNA data indicated that both cells were infected with approximately equal levels of virus.

To determine the potential effects of Hsp16 on viral replication, the Vpr-positive and Vpr-negative matching infectious pNLHX clones (57) were used to infect CD4⁺ H9 and CEM-SS cells. Equal levels of infection of the cells were verified by measuring viral RNA levels 24 h after viral infection by using the Roche Monitor assay according to the manufacturer's instructions. Viral replication was determined in CD4⁺ H9 and CEM-SS cells by determining p24 antigen levels with a commercially available HIVAG-1 polyclonal antigen kit (Abbott Laboratories, Abbott Park, Ill.).

Measurement of the Vpr-specific activities in fission yeast. All of the functional assays used to measure Vpr-specific activities, i.e., cell cycle G₂ arrest, nuclear localization, and cell death induced by Vpr, have been described previously (9, 60, 65). Vpr-induced cell elongation, which is commonly known as the "cdc phenotype" (Fig. 1A) (19, 28, 35, 39), is the result of cell cycle G₂ delay. As described previously (19), this cell elongation phenotype was used as an initial marker for Vpr-induced G₂ arrest in this study. Cell images were first captured on a Leica microscope, and cell length was measured individually with the

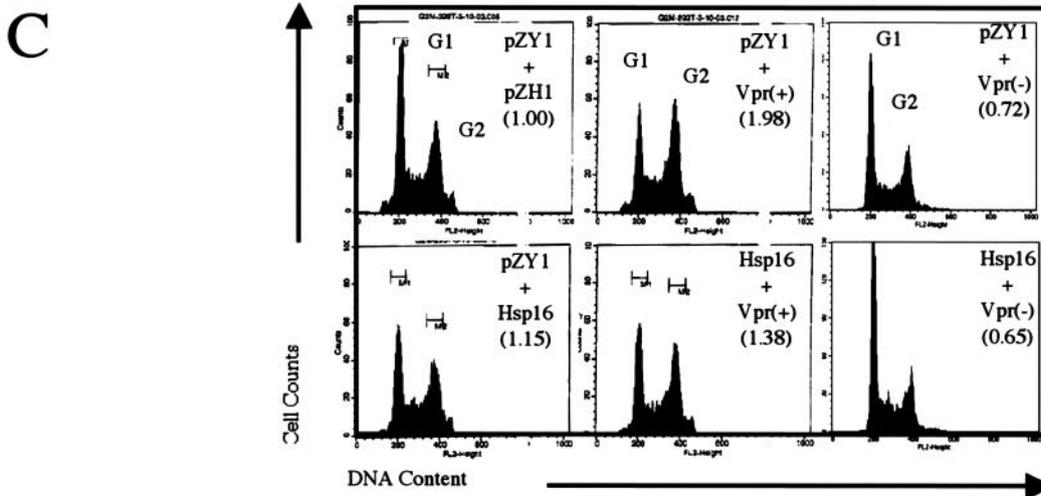
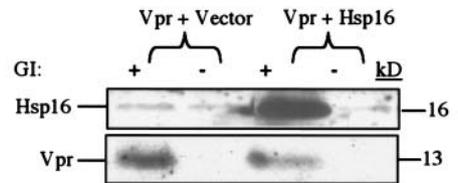


pZY1
+
pZH1

Vpr
+
pZH1

Vpr
+
Hsp16

b



captured images by using OpenLab software according to manufacturer's instructions. The statistical significance of cell length variation under different conditions was determined by using the *t* test for paired two samples for means. For example, in thiamine-containing growth medium (*vpr* gene expression is off [*vpr*-off]), fission yeast cells with a *vpr* plasmid are of normal length, with an average length of 8 to 12 μm (64), but in thiamine-free medium (*vpr*-on), cells average 15 to 18 μm in length after approximately 24 to 30 h of gene induction (19). As a result, *vpr*-expressing cells either stop cell division when the wild-type *vpr* (RE007) is expressed (60) or show a delay in entering mitosis when the mutant F34I*vpr* (RE076) is expressed (19). The effect of Vpr on cell proliferation can be further determined by growth kinetics analyses (60). Colony formation on agar plates was used as a measure of Vpr-induced cell death, which has been described previously (65). Briefly, *S. pombe* cells containing the pYZIN::*vpr* plasmid were first grown on a selective leucine-free EMM plate under *vpr*-repressing conditions. A loopful of viable cells was then streaked onto *vpr*-inducing or *vpr*-repressing EMM plates and incubated at 30°C for 3 to 4 days. No colonies or small colonies on the *vpr*-inducing EMM plates and normal growth on the *vpr*-repressing EMM plates indicate Vpr-induced cell killing (9, 63). Nuclear localization of Vpr was followed with a green fluorescent protein (GFP)-tagged Vpr (9, 65). Cultures of cells with plasmids expressing GFP or the GFP-Vpr fusion proteins were prepared the same way as described above for the measurement of G₂ arrest. Cellular localization was determined 18 to 24 h after *vpr* gene induction on a Leica fluorescence microscope.

Measurement of the Vpr-specific activities in mammalian cells. All mammalian transfections were conducted by electroporation on a Bio-Rad Gene Pulser II, according to the manufacturer's protocols. A total of 5 to 10 μg of DNA was normally used per transfection. To test the potential effects of the Vpr suppressors on nuclear membrane localization and transport of Vpr, a 1:9 ratio of the *gfp-vpr* carrying plasmid to the suppressor gene-carrying plasmids was used to maximize the suppressor gene expression in each of the *vpr*-expressing cells. Cells were washed with PBS 24 h after transfection, fixed on coverslips for 10 min in 4% paraformaldehyde, and rinsed in PBS. The coverslips were then inverted and mounted on glass slides with mount medium H1200 containing DAPI (4',6'-diamidino-2-phenylindole) (Vector Laboratory Inc). To measure nuclear import of Vpr, 400 nM leptomycin B (LMB) was added to the medium for 1 h prior to cell fixation according to a procedure previously described (45). It is of note that a wide range of LMB concentrations from nanomolar to micromolar could be used to inhibit the nuclear export of proteins. Even though a concentration as low as 5 nM LMB has been used to block nuclear export of a GFP-pyruvate kinase (PK)-Vpr fusion protein (45), we used 400 nM here to ensure nuclear localization of Vpr.

To measure Vpr-induced G₂ arrest in *vpr*-inducible 293T cells or cells infected with VSV-G-pseudotyped HIV-1, flow cytometric analysis was carried out as previously described with minor modifications (66). Briefly, approximately 10⁶ 293T-632 cells transfected with both pZH1-*vpr* and pZY1-carrying genes of interest were plated into 25 cm² flasks and grown under selection by zeomycin (400 $\mu\text{g}/\text{ml}$), Geneticin (750 $\mu\text{g}/\text{ml}$), and hygromycin (200 $\mu\text{g}/\text{ml}$) for 5 to 7 days prior to *vpr* gene induction. Muristerone A (1.0 μM) was added to cell cultures to induce gene expression as previously described (66). Cells were collected in 1 ml of PBS with 5 mM EDTA, fixed by the addition of 2.5 ml of cold 100% ethanol, and stored at 4°C for at least 15 h. The cells were washed twice with PBS-5 mM EDTA and treated with RNase A (2 $\mu\text{g}/\text{ml}$) at 37°C for 30 min prior to propidium iodide staining. Propidium iodide was added to a final concentration of 10 $\mu\text{g}/\text{ml}$, and flow cytometric analysis was carried out after a 1-h incubation on ice. The DNA content of the transfected cells was determined on a Becton-Dickinson flow cytometer by using Cellquest software, and cell cycle profiles were calculated and drawn by using a ModFit LT 3.0 program (Varity Software House). Vpr-induced cell death in HIV-infected CD4⁺ H9 cells was evaluated either by trypan blue staining, a dye that specifically recognizes dead

cells (Sigma), or with a commercial colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] assay, which measures cell viability, used according to the manufacturer's instructions (Boehringer Mannheim).

Searches for multicopy Vpr suppressors in fission yeast. To search for multicopy suppressors of Vpr-induced G₂ arrest, an *S. pombe* expression cDNA library was transformed into the RE076 strain, which carries a single integrated copy of F34I*vpr* (19). The use of RE076 in measuring Vpr-induced G₂ arrest is based on the fact that a single amino acid substitution at residue 34 of Vpr (F34I) eliminates *vpr*-induced cell death but does not affect the induction of cell cycle G₂ delay in either mammalian or fission yeast cells (9, 19, 54). Vpr-induced cell elongation in RE076 indicates a G₂ arrest and was used as an initial marker for the screens. Genes causing a reduction of cell length back to the normal length or shorter (i.e., <12 μm) in the *vpr*-expressing RE076 strain were considered as potential suppressor candidates. Out of approximately 2.0 \times 10⁴ transformants individually screened under the microscope, a limited number of Vpr suppressor candidates were identified and characterized (19). The suppression effects were confirmed by reintroducing the corresponding cDNA-carrying plasmids back into the parental strain, and the putative gene functions were identified by BLAST homology searches of *S. pombe* genome databases.

A similar strategy was also used to screen for multicopy suppressors of Vpr-induced cell death or nuclear localization of Vpr. For each screen, more than 2.0 \times 10⁴ transformants were screened and tested. Strains with a single integrated copy of the wild-type *vpr* (RE007) or *gfp-vpr* (RE078) were used as the tester strains for these two Vpr activities. The criterion used to identify suppressors of Vpr-induced cell death was the ability of a fission yeast transformant to form colonies of normal size on the *vpr*-expressing agar plate as previously described (60, 63). Potential Vpr suppressors for nuclear localization were individually screened under the fluorescent microscope by looking for displacement of Vpr from its localization on the nuclear membrane.

Fluorescence microscopy. A Leica DMR fluorescence microscope equipped with a high performance charge-coupled device camera (Hamamatsu) and OpenLab software (Improvision, Inc., Lexington, Mass.) was used for all imaging analysis. For the observation of GFP, we used a Leica L5 filter, which has an excitation of 480 nm (range, 460 to 500 nm) and emission of 527 nm (range, 512 to 542 nm). For DNA staining, cells were counterstained with 1 $\mu\text{g}/\text{ml}$ DAPI, which was observed with a Leica A8 filter with an excitation of 360 nm (range, 340 to 380 nm) and emission of 470 nm (range, 450 to 490 nm).

Other molecular and cellular techniques. The induction of cellular heat shock responses was conducted as previously described (26, 49). Briefly, cultures were first grown as mentioned above to fully express Vpr and then exposed to either an acute heat shock at 45°C for 15 min or grown at high temperature (36°C) for the indicated period of time. Coimmunoprecipitations were conducted by essentially following a standard procedure (<http://www.bio.uva.nl/pombe/handbook>).

RESULTS

Suppression of Vpr-induced cell cycle G₂ arrest by overexpression of fission yeast small heat shock protein Hsp16. To understand the molecular mechanisms underlying the ability of Vpr to induce cell cycle G₂ arrest, we launched a genome-wide screening for multicopy suppressors of Vpr-induced G₂ arrest. The screen was carried out in fission yeast strain RE076 carrying a single integrated copy of an inducible F34I*vpr* gene in the *S. pombe* chromosome (19) (Table 1). As shown in Fig. 1A (panel a, top row), expression of F34I*vpr* in *S. pombe* strain RE076 induced the cell elongation typical of a cell cycle G₂

FIG. 1. Overexpression of *hsp16* suppresses Vpr-induced cell cycle G₂ arrest in fission yeast and human noninfected and HIV-infected cells. (A) Suppression of Vpr-induced G₂ arrest by Hsp16 as measured in fission yeast by Vpr-induced cell elongation (a) and growth delay (b) in RE076 cells. Cell length was measured after 21 h of gene induction at 30°C. Growth kinetics was measured at 25°C. (B) Suppression of Vpr-induced G₂ arrest by Hsp16 in human 293T cells. (a) The extent of Vpr-induced G₂ arrest, measured by flow cytometric analysis, was quantified by the relative G₂ to G₁ ratio between gene-repressing and gene-expressing cells 72 h after gene induction. A relative G₂/G₁ ratio close to 1 indicates no significant difference between the gene-on and gene-off cultures. A relative ratio of >1 suggests increased G₂ cells in the gene-on culture (66). (b) Western blotting shows specific expression of HIV-1 *vpr* and the fission yeast *hsp16* gene in 293T-632 cells. GI, gene induction. (C) Suppression of Vpr-induced G₂ arrest by Hsp16 in 293T cells infected with VSV-G pseudotyped HIV-1_{NL4-3}. Vpr-positive or Vpr-negative HIV-1_{NL4-3} (57) was used to infect *hsp16*-expressing cells. Cell cycle profiles were measured by flow cytometric analysis 48 h after viral infection. Cells were infected with viruses adjusted to 3 \times 10⁶ cpm of RT activity per 10⁶ cells.

delay, which is commonly known as the *cdc* phenotype (19, 28, 39). Cell length measurements 21 h after gene induction indicated that *vpr*-expressing cells had an average length of $17.2 \pm 4.8 \mu\text{m}$ ($n = 200$) during log phase growth (19), which is significantly longer ($P < 0.001$) than normal cells and *vpr*-repressing cells, with an average length of $12.3 \pm 2.5 \mu\text{m}$ (Fig. 1A, panel a, top left) (19). However, the Vpr-induced G_2 delay was strongly suppressed when a cDNA clone encoding *hsp16* was expressed from the *nmt1* promoter in the same *vpr*-expressing RE076 strain (Fig. 1A, panel a, bottom rows). Coexpression of *vpr* with *hsp16* resulted in no significant increase ($P = 0.124$ for two-tails; $n = 200$) in cell length compared to the lengths of cells without expression of the *hsp16* gene. Suppression of Vpr-induced cell cycle delay by *hsp16* was further confirmed by its ability to prevent Vpr-induced growth delay, a result of the G_2 delay (Fig. 1A, panel b) (60, 65). A normal *vpr*-repressing cell culture grown at 25°C reaches stationary phase after about 45 h with a doubling time of about 3 to 4 h. In contrast, a significant growth delay was observed when the *vpr* gene was fully expressed 20 h after initiating gene induction in the thiamine-depleted (*vpr*-expressing) medium. The difference in growth rate was more than 1 log unit, and the *vpr*-expressing cells did not reach stationary phase until 60 h later (Fig. 1A). In contrast, this growth delay was eliminated when *hsp16* was expressed in the *vpr*-expressing RE076 cells. The growth kinetics of these two cultures were very similar to the kinetics of the cell cultures without *vpr* gene expression, indicating that expression of this small heat shock protein suppressed the growth inhibition caused by Vpr.

To test whether suppression of Vpr-induced G_2 arrest by Hsp16 is conserved in mammalian cells, we expressed Hsp16 in 293T cells. To measure the Vpr-induced G_2 arrest in mammalian cells, we adapted an inducible *vpr* gene expression system in a human embryonic kidney 293T-632 cell line (66). In this system, *vpr* gene expression is induced by adding $1.0 \mu\text{M}$ muristerone A (66). As shown in Fig. 1B (panel a), addition of muristerone A to the control cell line 293T-632 not containing Vpr did not affect the cell cycle profile 72 h after gene induction (G_2/G_1 ratio of 1.08 ± 0.5). In contrast, a significant increase in the G_2/G_1 ratio (2.15 ± 0.47) was observed when the *vpr* gene was expressed, indicating an accumulation of G_2 cells in the *vpr*-expressing cultures (66). Coexpression of fission yeast *hsp16* together with *vpr* blocked Vpr-induced G_2 arrest (Fig. 1B, panel a). A relative G_2/G_1 ratio of 0.98 ± 0.45 was observed for the Hsp16 culture. Western blot analysis showed that both Vpr and Hsp16 were properly produced and that Hsp16 did not significantly affect the protein level of Vpr, indicating a specific suppression of Vpr-induced G_2 arrest by Hsp16 (Fig. 1B, panel b).

To test whether the suppressive effects of Hsp16 on Vpr-induced G_2 arrest observed in fission yeast and mammalian cells also applies in the context of viral infection, we used VSV-G-pseudotyped HIV-1 viruses to infect 293T cells (Fig. 1C) (8, 57). Both Vpr-positive and Vpr-negative viruses used for viral infection are isogenic except that Vpr-negative virus carries a single base mutation at the start codon of the *vpr* open reading frame which changes ATG to GTG (43). The 293T cells that stably expressed the control vector pZY1 (top row) or Hsp16 (bottom row) were infected. As shown in Fig. 1C, coexpression of the vectors pZY1 and pZH1 did not affect the

cell cycle profile of 293T cells. Infection with Vpr-positive virus caused a significant shift of the G_2/G_1 ratio to 1.98, indicating a cell cycle G_2 shift due to viral infection (Fig. 1C). In contrast, infection of the same 293T cells with Vpr-negative virus did not affect the cell cycle profile (top right), indicating that the G_2 shift was caused specifically by Vpr. Consistent with our findings in fission yeast and mammalian cells, with overproduction of Hsp16 (bottom middle) the cell cycle profile at least in part reverted back to normal, with a relative G_2/G_1 ratio of 1.38. Together, these data suggest that the suppression of Vpr-induced cell cycle G_2 arrest by Hsp16 is a conserved activity between human and fission yeast cells.

Suppression of the Vpr-induced G_2 arrest by Hsp16 mimics cellular heat shock response. We next investigated the molecular mechanism underlying the inhibiting effects of Hsp16 on Vpr. Since the mRNA levels of Hsp16 increase up to 60-fold when *S. pombe* cells are subject to heat shock (49), we investigated whether heat shock could suppress Vpr. Vpr-expressing RE076 cells were first grown at 30°C for 21 h to allow full expression of *vpr*. Cells were then heat shocked at 45°C for 15 min, and both the treated and untreated cells were allowed to recover for three additional hours after heat shock treatment at 30°C prior to observation. Consistent with our earlier findings (Fig. 1A, panel a) (19), expression of *vpr* in the untreated controls induced longer cells in comparison to the non-*vpr*-expressing cells (Fig. 2A, panel a). In contrast, no significant difference was found between the *vpr*-on and *vpr*-off cells in the heat-treated cell cultures. Tests of cell viability indicated that the brief heat treatment of *S. pombe* cells at 45°C did not comprise cell viability. Thus, these data suggest that heat shock, indeed, suppresses Vpr-induced cell elongation (Fig. 2A, panel a). However, this acute heat shock caused only a transient effect on Vpr activity, as the heat-treated *vpr*-expressing cells became longer after extended incubation at 30°C (data not shown). To test whether constant high temperature incubation would sustain this suppression, a time course growth experiment was conducted (Fig. 2A, panel b). While expression of *F34lvpr* induced a growth delay at 25°C , this delay was completely eliminated when the same *vpr*-expressing cells were grown at 36°C (Fig. 2A, panel b). Western blot analysis further showed that Vpr proteins were properly produced in the heat-treated RE076 cells at a level similar to that in the untreated cells, indicating that Vpr protein production was unaffected by heat shock (Fig. 2B). Thus, suppression of the Vpr-induced G_2 arrest by Hsp16 indeed mimics the cellular heat shock response.

Suppression of Vpr-induced cell death by overexpression of Hsp16. Following the G_2 arrest induced by Vpr, cells undergo a process of cell death (46, 48, 65). Many studies have reported that Vpr induces apoptosis in human cells (3, 48, 59). However, Vpr seems to induce more cell death than can be accounted for solely by apoptosis (59). Indeed, Vpr-induced cell death has been shown to be either mitochondrial dependent (46, 48) or mitochondrial independent (29, 59, 62, 65). We therefore investigated whether Hsp16 can protect cells from the overall cell death caused by Vpr.

In fission yeast, Vpr-induced cell death can normally be detected by the inability of *vpr*-expressing cells to form colonies on an inducing agar plate (9, 65). For example, cells without *vpr* gene expression (*vpr*-off) grow normally on a repressing

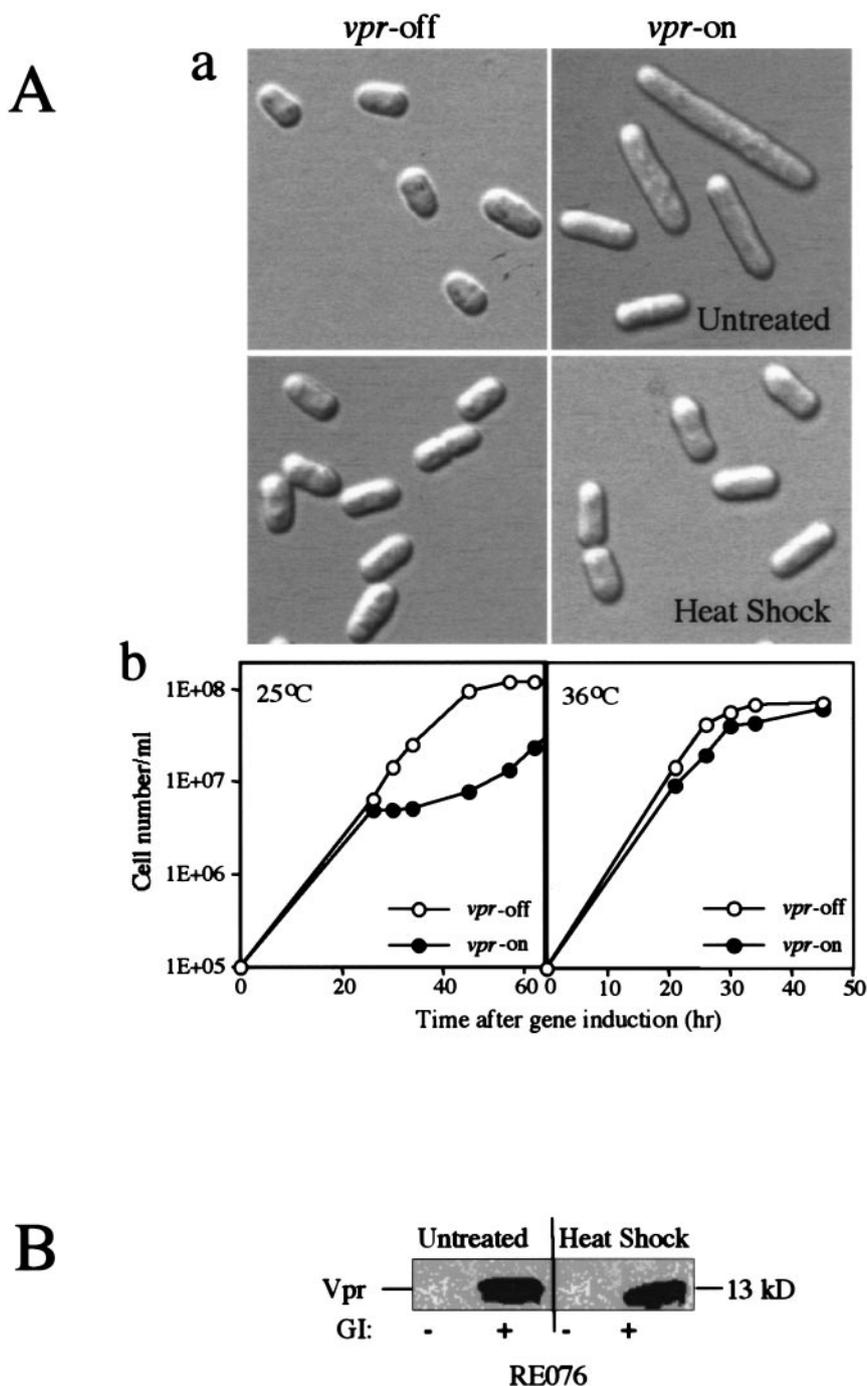


FIG. 2. Suppression of Vpr-induced cell cycle G_2 arrest by Hsp16 mimics cellular heat shock responses. (A) Induction of cellular heat shock responses suppresses Vpr-induced G_2 arrest as indicated by prevention of the Vpr-induced cell elongation (a) and growth delay (b). Cellular heat shock responses were triggered either by an acute heat treatment (45°C for 15 min) or constant high temperature at 36°C. (B) Induction of cellular heat shock responses does not affect the protein levels of Vpr as indicated by Western blot analysis. GI, gene induction.

plate (Fig. 3A, panel a). In contrast, only very small colonies or no colonies could be seen on an inducing plate where *vpr* is expressed (Fig. 3A, panel a). Earlier studies showed that the absence of colonies is due to rapid induction of cell death by wild-type Vpr (60, 63). However, when *hsp16* was coexpressed with *vpr*, the cells regained their ability to form colonies of

normal size, indicating that overexpression of *hsp16* suppressed Vpr-induced cell death (Fig. 3A, panel a). Consistent with this observation, we have also identified *hsp16*, through an independent screen for multicopy suppressors, as a strong suppressor for Vpr-induced cell death (data not shown). Moreover, suppression of Vpr-induced cell death by Hsp16 was

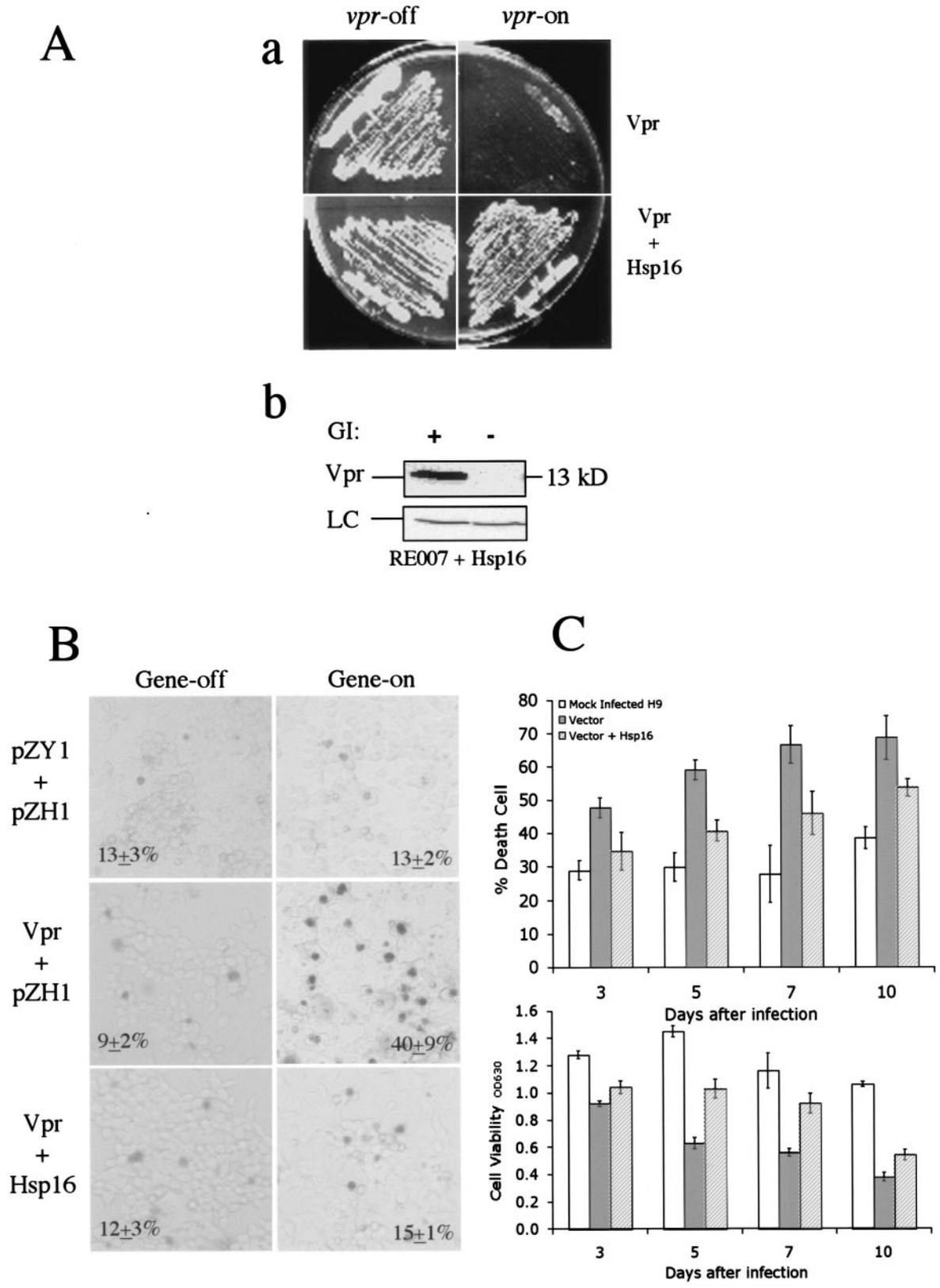


FIG. 3. Overexpression of *hsp16* suppresses Vpr-induced cell death in fission yeast and human noninfected and HIV-infected cells. (A) Suppression of Vpr-induced cell death in RE007 cells as shown by colony forming ability on EMM selective agar under the *vpr*-inducing (*vpr-on*) and *vpr*-repressing (*vpr-off*) conditions (a). Plates are shown after 3 to 4 days of incubation at 30°C. Overproduction of Hsp16 in RE007 does not affect the protein levels of Vpr as detected by anti-Vpr serum in a Western blot analysis (b). RE007 cells expressing *hsp16* were collected 24 h after gene induction at 30°C. GI, gene induction; LC, protein loading control. (B) Suppression of Vpr-induced cell death by Hsp16 in human 293T cells. Dead

specific as overexpression of another *S. pombe* small heat shock protein, *hsp9*, did not restore colony formation (data not shown). To test whether Hsp16 interferes with the expression of the *vpr* gene, Western blot analysis was performed to determine the Vpr protein levels in *hsp16*-expressing cells. While no Vpr was detected when the *vpr* gene was repressed in thiamine-containing medium (Fig. 3A, panel b), a band corresponding to the Vpr 13-kDa protein was found in cells coexpressing *hsp16* (Fig. 3A, panel b), indicating Vpr production was not affected by Hsp16.

A similar suppressive activity was also observed when Vpr-induced cell death was evaluated in mammalian 293T cells by staining dead cells with trypan blue (Fig. 3B). Cell death in the *vpr*-expressing cells was examined 72 h after *vpr* gene induction. A minimal amount of cell death (9 to 13%) was observed in all of the cell cultures without *vpr* gene induction (Fig. 3B). A similar background level of cell death ($13\% \pm 2\%$) was also observed in the 293T-632 control cells treated with 1.0 μM muristerone A (top right). In contrast, a significant increase in dead cells staining with trypan blue ($40\% \pm 9\%$) was found in the *vpr*-expressing cell culture (Fig. 3B, second row). However, cell death was reduced to near background levels ($15\% \pm 1\%$) when *hsp16* was coexpressed with *vpr*. Thus, expression of fission yeast *hsp16* appears to suppress Vpr-induced cell death in human 293T cells (Fig. 3B, bottom right).

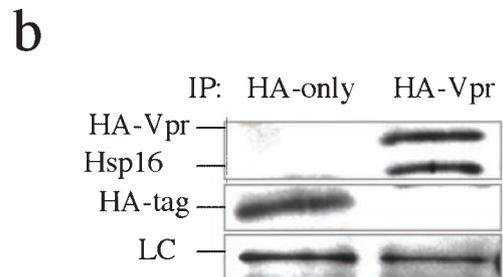
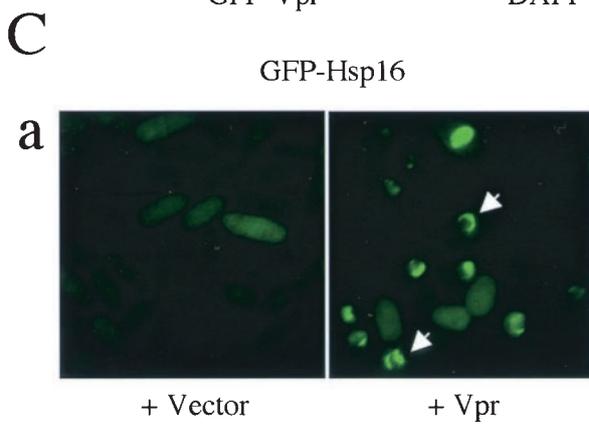
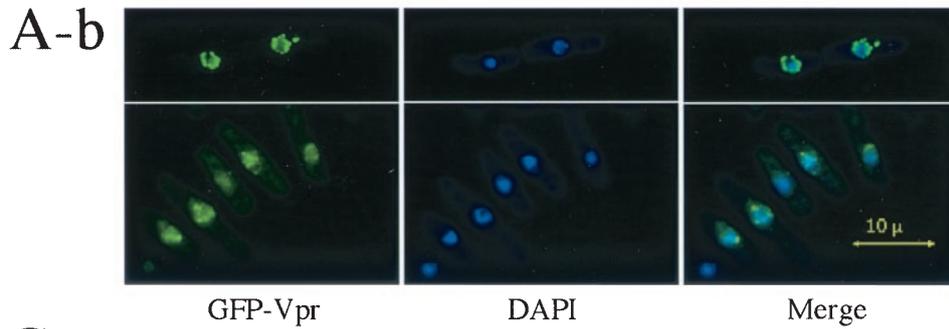
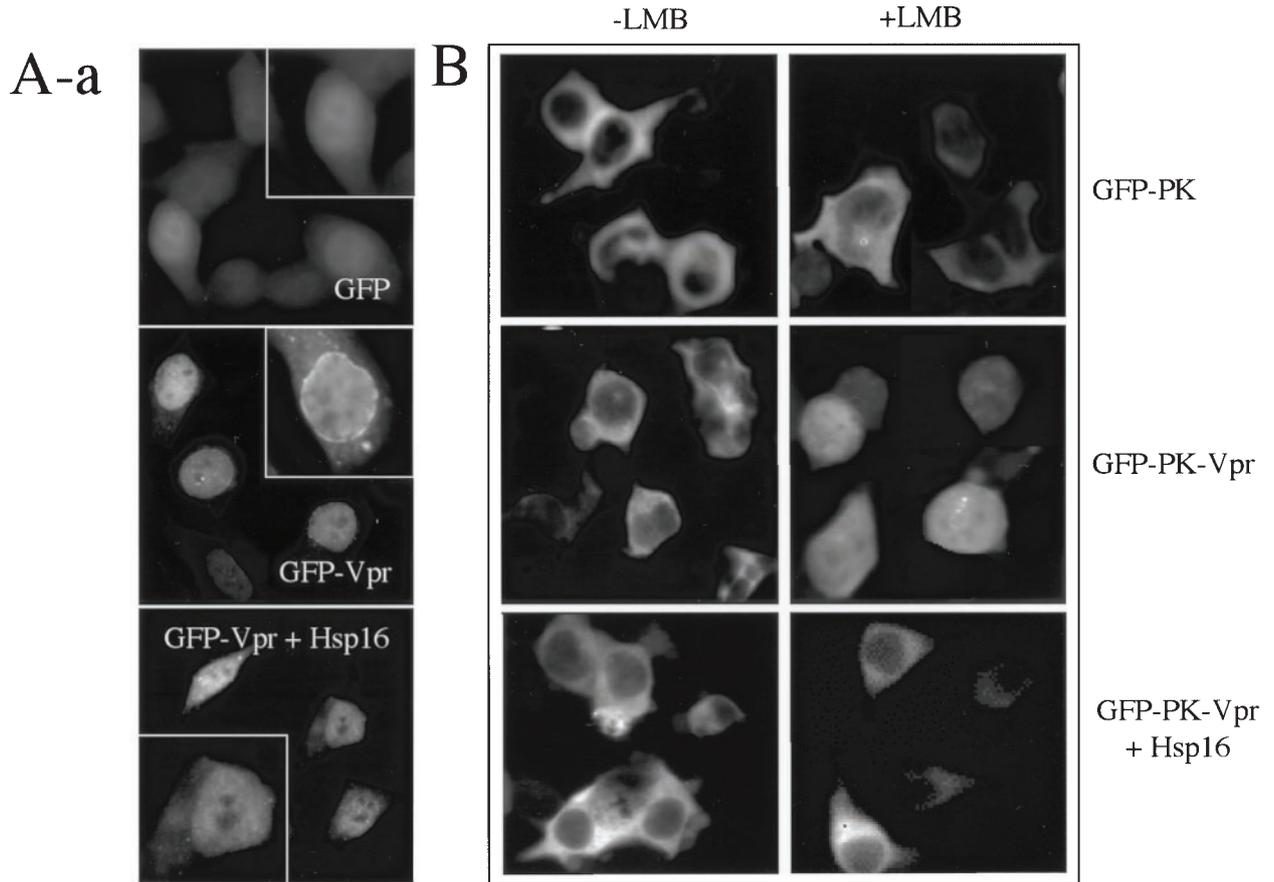
To evaluate the potential suppressive effect of Hsp16 on cell killing caused by HIV infection, CD4⁺ H9 cells were mock infected (Fig. 3C) or infected with HIV-1_{LAI} plus a vector control or HIV-1_{LAI} plus the vector expressing *hsp16*. Equal levels of HIV-1 infection of these cells were confirmed by determination of viral RNA levels 24 h after infection. The viral RNA loads were 7.65×10^5 copies/ml for H9 without Hsp16 and 6.92×10^5 copies/ml for H9 cells with Hsp16, respectively. Since variations of the Roche HIV-1 monitor assay are normally within threefold, the viral RNA load data indicated that both cell groups were infected with approximately equal numbers of viruses. Cell death caused by HIV-1 was evaluated 3, 5, 7, and 10 days after viral infection by staining with trypan blue (Fig. 3C, top) or by measuring cell viability with the MTT assay (Fig. 3C, bottom). Significant suppression of cytotoxicity caused by HIV-1 infection was observed in cells that stably produced Hsp16. For example, the background cell death ranged from 29 to 38% in mock-infected cells (Fig. 3C). A steadily increasing percentage of dead cells (47 to 69%) was observed in HIV-infected cells with the control vector from day 3 to day 10 after viral infection. In contrast, an approximately 27% reduction in cell death was observed in the *hsp16*-expressing cells 3 days after viral infection, and a 22 to 31% reduction was observed from 5 to 10 days after viral infection. Similar suppression by Hsp16 was also found when the MTT assay was used to determine cell viability (Fig.

3C). For example, cell viability decreased from 28 to 64% from day 3 to day 10 after viral infection. From 30 to 39% more viable cells were recovered in *hsp16*-expressing cells at 5 to 10 days even though there was no significant recovery of cell viability at 3 days. Thus, overproduction of Hsp16 confers at least partial protection in the context of viral infection.

Hsp16 interferes with nuclear membrane localization and nuclear import of Vpr by direct protein-protein interaction. HIV-1 Vpr localizes at higher levels on the nuclear membrane relative to nucleus and cytoplasm in both yeast and mammalian cells (9, 54). The biological significance of Vpr accumulation on the nuclear membrane is at present unclear. Earlier studies demonstrated that Vpr binds to importin α on the nuclear membrane (42, 54), indicating a potential role for Vpr in nuclear targeting, possibly by regulating the docking of the pre-integration complex to the nuclear pore complex. Earlier studies showed that HSP70 competes with Vpr for its binding to importin α (2) and that overexpression of HSP70 displaces Vpr from the nuclear membrane (unpublished data). This raises the possibility that Hsp16 may also be able to displace Vpr from the nuclear membrane. To test this possibility, GFP-tagged Vpr was used to monitor Vpr localization onto the nuclear membrane in HeLa cells (58). When the *gfp* gene was expressed by itself in HeLa cells, a relatively uniform GFP distribution was observed throughout the cells, with somewhat stronger nuclear accumulation. However, the nuclear rim was not clearly visible (Fig. 4A, panel a, top). In contrast, an easily distinguishable nuclear membrane localization of GFP-Vpr was detected even though Vpr was also detected in the cytoplasm and nucleus similar to GFP alone (panel a, middle). Consistent with earlier findings (54, 56), this observation indicates preferential localization of some Vpr to the nuclear membrane (54, 58). However, when *hsp16* was coexpressed with *vpr*, the unique nuclear rings formed by GFP-Vpr disappeared (panel a, bottom), suggesting that Hsp16 may be able to prevent Vpr localization to the nuclear membrane in HeLa cells. Consistent with these observations, we have independently identified the same effect of Hsp16 on Vpr in fission yeast cells through a genome-wide screening for multicopy suppressors of Vpr localization on the nuclear membrane (Fig. 4A, panel b). In fission yeast cells, Vpr localizes almost exclusively to the nuclear rim with little cytoplasmic distribution and with the center of nucleus clear (Fig. 4A, panel b, top) (9). As shown in Fig. 4A (panel b, bottom), overexpression of *hsp16* prevented localization of Vpr on the nuclear membrane; instead, it localized within the nucleus and also had somewhat increased distribution throughout the cytoplasm. Together, these observations suggest that Hsp16 may interfere with the nuclear localization of Vpr onto the nuclear membrane.

Since Vpr cycles between the nucleus and cytoplasm in mammalian cells (15, 45), the increased cytoplasmic localiza-

cells were detected 72 h after gene induction by trypan blue staining. Gene-off, no muristerone A was added; Gene-on, 1.0 μM muristerone A was added to induce gene expression. (C) Suppressive effects of Hsp16 on cytopathicity induced by HIV-1 infection in H9 cells. Top panel shows cell death induced by HIV-1 infection. Bottom panel measures cell viability during the course of the experiments. H9 cells were either mock infected (left columns) or HIV-1_{LAI}-infected cells carrying empty vector (middle columns) or a vector expressing *hsp16* (right columns). Cell death and viability of the infected cells were examined 3, 5, 7, and 10 days after infection. The percentage of cell death induced by HIV-1 infection was quantified by trypan blue staining. Cell viability was determined by measuring the optical density at 630 nm (OD₆₃₀) by using a commercial MTT assay (Boehringer Mannheim).



tion of Vpr in cells expressing Hsp16 could either be due to the enhancement of nuclear export or to the inhibition of nuclear import of Vpr. To distinguish these two possibilities, a GFP-PK-Vpr fusion protein system was used to monitor nuclear import or export of Vpr (45). In this system, LMB was used to block nuclear export of Vpr. A wide range of concentrations of LMB (from nanomolar to micromolar) has been used in various studies to examine nuclear export of proteins. A concentration as low as 5 nM LMB was shown to block nuclear export of Vpr (45). We used 400 nM to ensure nuclear localization of Vpr constructs. A GFP-PK fusion protein was used as a control since it does not enter the nucleus regardless of LMB treatment (Fig. 4B). The GFP-PK-Vpr fusion, on the other hand, accumulates in the nucleus upon the LMB treatment (Fig. 4B, middle row, right), thus providing a specific assay for measuring nuclear import of Vpr (45). By using this assay, we tested the possible effect of Hsp16 on the nuclear import of Vpr. Relatively clear nuclei indicating low levels of GFP-PK-Vpr were found when the fission yeast *hsp16* was coexpressed in the LMB-treated cells, suggesting that expression of Hsp16 at least partially suppressed the nuclear import of Vpr (Fig. 4B, bottom).

Even though overexpression of *hsp16* is able to block the accumulation of Vpr on the nuclear membrane, Hsp16 by itself normally distributes uniformly throughout the fission yeast cell at a low level in the absence of heat shock (Fig. 4C, panel a, left) (49). To examine the cellular distribution of Hsp16 in the presence of Vpr, we expressed *vpr* in an *S. pombe* strain (Q1649) in which the *hsp16* gene is tagged with GFP and integrated as a single-copy gene into the chromosome (49). In contrast to the uniform subcellular distribution of Hsp16 observed without Vpr, under these conditions an accumulation of Hsp16 is now seen close to the nuclear membrane where Vpr localizes (Fig. 4C, panel a, arrows). This mutual redistribution of Vpr and Hsp16 suggests a potential interaction between these two proteins. To test this possibility, immunoprecipitation was carried out from cells transformed with either a hemagglutinin (HA)-tagged Vpr or a control (HA-tag) by using anti-HA antibody, and both the anti-HA and anti-Hsp16 antibodies were used to detect the coimmunoprecipitation of these two proteins. As shown in Fig. 4C (panel b), a small band corresponding to the HA-tag was found reacting with the anti-HA antibody when only HA-tag was used as a control. Neither Vpr nor Hsp16 was detected in this pull down. However, both Hsp16 and HA-tagged Vpr were detected in the HA-Vpr

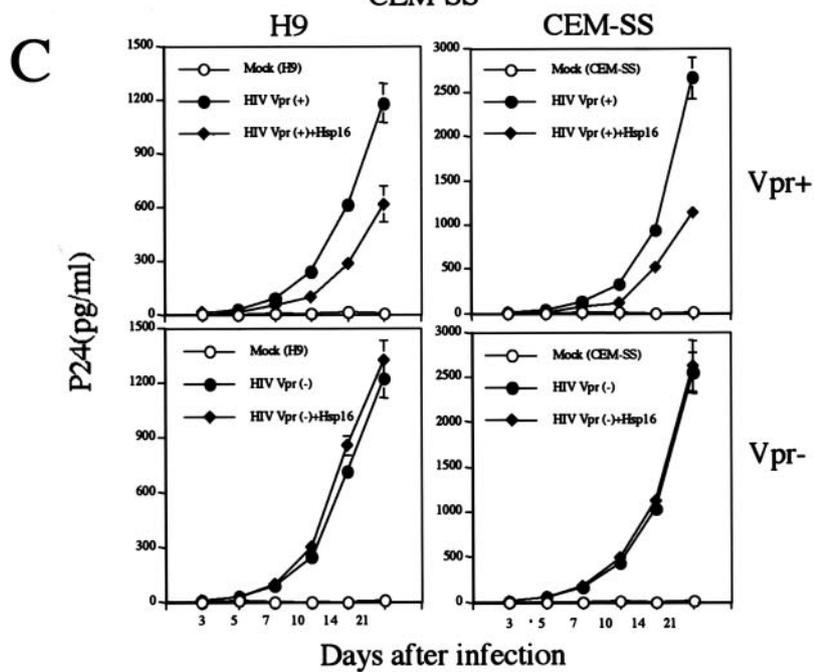
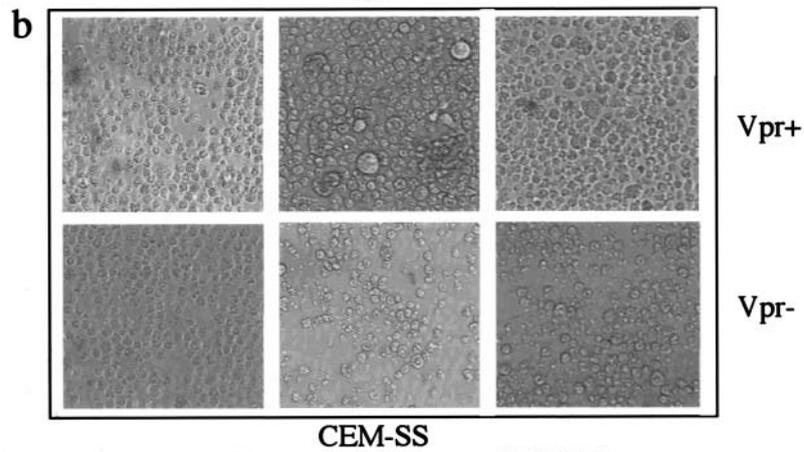
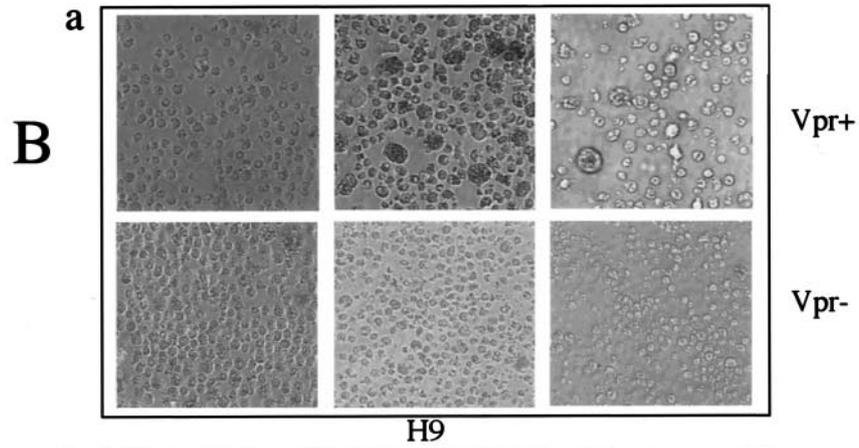
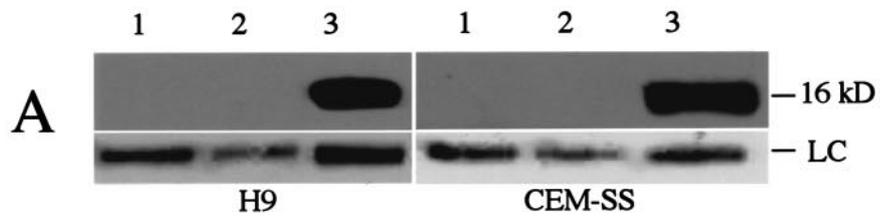
pull-down products, indicating the association of these proteins within the cell. Together, these observations suggest that Hsp16 interferes with nuclear membrane localization and nuclear import of Vpr through direct protein-protein interaction.

Hsp16 inhibits viral replication in a Vpr-dependent manner.

Vpr activities such as G₂ arrest and nuclear targeting to the nuclear membrane have been implicated as positive factors for HIV-1 replication (16, 22, 23), and consistent with these activities, Vpr has been shown to increase viral replication up to fourfold in some proliferating T lymphocytes (22, 53, 59). Because Hsp16 counteracts some of these Vpr activities, we expected that Hsp16 might be able to inhibit HIV-1 replication at a comparable level. To test this possibility, we established CD4⁺ H9 and CEM-SS cell lines stably producing high levels of Hsp16 (Fig. 5A). These H9 or CEM-SS cells were then infected with two matched pNLHX viral clones (55) that differ only in the *vpr* gene. The Vpr-negative construct has a single base mutation at the start codon of the *vpr* open reading frame, which changes ATG to GTG (42). Equal levels of infection of the cells were verified by measuring viral RNA levels 24 h after viral infection by using the Roche Monitor assay. Results of these measurements showed that the viral RNA loads varied in the range of $\log 4.87 \pm 0.02$ copies/ml for H9 cells infected with Vpr-positive or Vpr-negative viruses, and $\log 5.05 \pm 0.02$ copies/ml for CEM-SS cells infected with Vpr-positive or Vpr-negative viruses, respectively. The viral load data indicated that CEM-SS cells were infected with a viral load about 1.5-fold greater than that in H9 cells. However, within each cell type an approximately equal amount of Vpr-positive or Vpr-negative virus was present. HIV-1 infection with virus carrying a wild-type *vpr* gene typically causes gross enlargement of the infected cells relative to the Vpr-negative virus (29), and transfection with just *vpr* alone is also sufficient to cause this enlargement (29, 33, 60). Consistently, H9 or CEM-SS cells infected with Vpr-positive viruses, indeed, appeared to be slightly larger than those infected with Vpr-negative cells (Fig. 5B), and the Vpr effect was especially indicated by a small percentage of extremely large cells (top middle). However, the number of these large cells was significantly reduced in the *hsp16*-expressing cells that were infected with Vpr-positive virus (top right). The observation that expression of *hsp16* was able to reduce formation of these large cells and restore nearly normal cell density (data not shown) suggests that Hsp16 does confer at least some degree of protection against Vpr.

To test the potential effect of Hsp16 on viral replication, p24

FIG. 4. Overexpression of *hsp16* interferes with nuclear membrane localization and nuclear import of Vpr through direct protein-protein interaction. (A) Hsp16 diminishes nuclear membrane localization of Vpr in HeLa cells (a) and fission yeast (b). Note that Vpr localization on the nuclear membrane in HeLa cells can be distinguished as a clear ring surrounding the nucleus in the GFP-Vpr panel. However, this nuclear ring formed by GFP-Vpr was not clearly visible in GFP alone or GFP-Vpr+Hsp16 panels. Single-cell inserts in each panel show a magnified view of the presence or absence of nuclear membrane localization of Vpr. Nuclear membrane localization of Vpr was visualized by the expression of the GFP-Vpr fusion protein 24 h after transfection in HeLa cells. Cells containing the pYZ1N vector show Vpr localization on the nuclear membrane (panel b, top); cells containing pYZ1N-*hsp16* show enhanced distribution of Vpr in the nucleus and cytoplasm (panel b, bottom). Nuclear membrane localization of Vpr was visualized 24 h after gene induction in fission yeast. DAPI staining was used to show nuclei. (B) Hsp16 blocks nuclear entry of Vpr in 293T cells. Nuclear import of Vpr was examined 24 h after transfection and 1 h after the addition of 400 nM LMB. (C) Potential interaction of Vpr with Hsp16. Panel a shows the effect of Vpr on subcellular localization of GFP-Hsp16 in strain Q1649. Note that expression of HIV-1 *vpr* changes the subcellular distribution of GFP-Hsp16 from evenly distributed (left) to a pattern that is similar to Vpr distribution, as indicated by arrows (right). Panel b shows the coimmunoprecipitation of Hsp16 with HA-tagged Vpr. Both anti-HA and anti-Hsp16 antibodies were used in Western blot analysis. LC, protein loading control.



antigen was measured in culture supernatants over a period of 14 to 21 days after infection. As shown in Fig. 5C, a consistent but moderate reduction of HIV-1 viral replication was observed in H9 cells expressing *hsp16*. For example, levels of p24 antigen steadily increased (from 26.0 ± 1.5 to $1,175.3 \pm 90.1$ pg/ml) in HIV-infected cells expressing the vector control from day 5 to day 21 of HIV-1 infection, indicating successful viral infection (Fig. 5C, top left). However, a 1.8- to 2.6-fold reduction in p24 antigen levels was detected in HIV-infected cells expressing Hsp16 during the same period after viral infection. No detectable p24 antigen was observed in mock-infected cells over the entire experimental period. To ensure that the observed viral inhibition by Hsp16 is not cell line-specific, we also examined CEM-SS cells, another CD4-positive cell line that was derived from T lymphocytes (37). A similar suppressive effect on viral replication (1.8- to 2.9-fold reduction) was observed in the CEM-SS cells that stably express *hsp16* genes (Fig. 5C, top right). It was noted that viral replication in CEM-SS cells was higher than in H9 cells. This may be due at least in part to the higher initial viral inoculum for CEM-SS cells.

To further examine whether the inhibitory effect of Hsp16 on viral replication is due to its suppression of Vpr activities or other viral factors, we repeated the same infection experiments in the H9 and CEM-SS cells by using a matching Vpr-defective viral clone that differs only in the *vpr* gene. The Vpr-negative construct has a single base mutation at the start codon of the *vpr* open reading frame, which changes ATG to GTG (42). As shown in Fig. 5C (bottom), the kinetics of viral replication were essentially indistinguishable between cells with or without Hsp16, suggesting that the inhibitory effect of Hsp16 on viral replication is specifically through Vpr and thus, Vpr-dependent.

DISCUSSION

A fission yeast small heat shock protein (Hsp16) was identified by three independent genome-wide searches for multicopy suppressors of Vpr nuclear localization and Vpr-induced cell cycle G₂ arrest and cell death. Cross-identification of this yeast protein in all three screens suggested that Hsp16 has a global suppressing effect on the Vpr activities. These suppressing effects seem to be a conserved phenomenon as Hsp16 showed similar suppressive activities toward Vpr in *S. pombe* and mammalian cells (Fig. 1, 3, and 4). Since Hsp16 inhibits all three Vpr activities that are functionally independent of each other (9, 18), the suppressing effect of Hsp16 might target either an early upstream step of the Vpr pathway or act directly on Vpr itself. Our data support the second possibility, as Hsp16 directly associates with Vpr (Fig. 4C, panel b). Hsp16 appears to have a stronger suppressive effect on Vpr in fission yeast

than in mammalian cells in the context of viral infection (Fig. 1C and 3C). While this may be related to differences in the levels of Hsp16 and Vpr in fission yeast compared to infection in mammalian cells, it is also possible that this yeast protein may not function in human cells in exactly the same way as in yeast cells. It will thus be of interest to test whether its human counterparts have similar but stronger suppression of Vpr during viral infection.

The suppression of Vpr activities by Hsp16 suggests that Hsp16 might inhibit viral replication, and Hsp16 was found to inhibit viral replication only in Vpr-positive virus (Fig. 5C). However, the inhibition of viral replication by Hsp16 appears to be too large to be accounted for simply by inactivation of Vpr. While a number of reports indicate that HIV-1 with Vpr replicates at 2 to 4 times higher levels in T-cell lines than virus without Vpr (22, 53, 59), others have reported that Vpr plays little or no role in the levels of viral replication in T-cell lines (12, 14, 21). The results shown in Fig. 5C indicate that, under these experimental conditions, Vpr has little effect (<20% decrease) on viral replication (compare the Vpr-negative virus in the bottom panel to the Vpr-positive virus in the top panel). This small decrease in the Vpr-negative virus replication is not sufficient to explain the $\geq 50\%$ decrease in viral replication after Hsp16 is present but only with Vpr-positive virus. One possible explanation is that the association of Hsp16 with Vpr does not simply inactivate Vpr per se but creates a complex that actively inhibits a step in viral replication. For example, Vpr is ordinarily packaged in the virus (11), but viruses with and without Vpr are apparently assembled with the same efficiency and are equally infectious (Fig. 5C) (12, 14, 21). If the Hsp16-Vpr complex is still recruited during virus assembly but this complex inhibits completion of viral assembly, then production of Hsp16 could inhibit replication via the Hsp16-Vpr complex although Vpr itself does not affect viral assembly or the efficiency of the replication step. Further work is necessary to establish whether the Hsp16-Vpr complex actively inhibits viral replication and, if so, which step or steps of replication are inhibited.

Suppression of Vpr by Hsp16 mimics the effects of cellular heat shock response because heat treatments of *vpr*-expressing cells suppressed the Vpr-induced growth delay (Fig. 2A), suggesting that Hsp16-mediated suppression of Vpr could be part of the cellular heat shock response. Heat shock proteins are well known for their molecular chaperone activities in protecting cellular proteins under stressful conditions. For example, small heat shock proteins have been shown to form large heteroaggregates and function as molecular chaperones to protect cellular proteins from denaturation in times of stress (31). The aggregation of small heat shock proteins associated with Vpr could prevent Vpr from interacting with other cellular proteins

FIG. 5. Stable expression of Hsp16 in H9 and CEM-SS cells prior to HIV infection reduces viral replication and HIV-induced cytopathicity in a Vpr-dependent manner. (A) Western blots show high levels of Hsp16 in CD4⁺ H9 and CEM-SS cells. Lane 1, mock-infected cells; lane 2, HIV-infected cells carrying plasmid pcDNA3.1; lane 3, HIV-infected cells expressing *hsp16*. Blots show 16-kDa bands reacted to anti-Hsp16 antibody. LC, protein loading control. (B) Stable expression of Hsp16 in H9 and CEM-SS cells blocks the gross enlargement of cells typically induced by Vpr-positive HIV infection (29). Images were taken with equal amount of cells. Gross enlarged cells are of typical effect caused by Vpr (29). (C) Stable expression of Hsp16 in CD4⁺ H9 and CEM-SS cells inhibits viral replication in Vpr-positive HIV infection; Hsp16 has no effect on viral replication in Vpr-negative HIV infection.

and thereby inhibit its activities. It is conceivable, however, that normal levels of the small heat shock proteins may not be sufficient to entrap Vpr, and thus overproduction of these small heat shock proteins either by expression from a strong promoter on a high-copy-number plasmid or by triggering cellular stress responses is needed to provide sufficient levels. This model is supported by our observations that there is indeed aggregated Hsp16 in *vpr*-expressing cells around the nuclear periphery where Vpr normally localizes (Fig. 4C, panel a, arrows). However, a more specific functional test such as measuring the chaperone activity of Hsp16 (34) is needed to further substantiate this possibility. It should be mentioned that this model does not explain the increased cytoplasmic localization of Vpr where no obvious Hsp16 aggregation was observed. An alternative and additional mechanism could be that Hsp16 may also inhibit Vpr activities by preventing entry of Vpr to the nucleus (Fig. 4B) (1, 2).

Human small heat shock protein HSP27 is a potential homologue of fission yeast Hsp16 as both proteins share a common C-terminal GXLX4P motif, a hallmark of the small heat shock proteins (10). In addition, anti-HSP27 antibodies weakly cross-reacted with Hsp16, suggesting a certain degree of similarity at the structural level between these two proteins (data not shown). Interestingly, earlier studies showed that HSP27 may play a specific role in resisting HIV infection (6, 7, 13). For example, expression of HSP27 is specifically elevated as early as 3 to 8 h after initial HIV infection (5, 55). Even though the molecular mechanisms underlying the HIV-responsive expression of these heat shock proteins have not been identified, it was surmised that HSP27 could participate in eliciting host innate antiviral immune responses, thus having protective effects against HIV infection (6, 7). Similarly, expression of *vpr* also elicits a moderate increase of Hsp16 both at translational and transcriptional levels in fission yeast (data not shown), suggesting that expression of this viral protein may be perceived as a stimulus to the activation of Hsp16. Consistent with the idea that the elevation of heat shock protein levels represents a cellular response to counteract the potential viral effect, here we show that overproduction of *hsp16* by heat treatment or gene overexpression suppressed Vpr-specific activities. Certainly, it is possible that the Vpr-Hsp16 interaction we observed in fission yeast may not represent what actually happens in mammalian cells. However, both the suppression of Vpr activities by Hsp16 and the heat shock responses appear to be highly conserved activities between *S. pombe* and mammalian cells, suggesting that a similar scenario, such as that described above for the HIV-HSP27 interaction, might occur in HIV-infected cells. Thus, it will be of interest to test whether HSP27 shows similar inhibitory effects against Vpr. Studies are under way in our laboratories to answer this question.

In summary, we have isolated a yeast chaperone protein that can specifically inhibit the activities of HIV-1 Vpr either as a single viral protein or in the context of viral infection. This is to our knowledge the first protein known to specifically inhibit Vpr in vitro and in vivo. This finding could potentially provide a new approach to study the specific effects of Vpr. The evidence presented here indicates that the suppressive effect of Hsp16 on the HIV-1 Vpr activities is a conserved cellular function, which may implicate a new level of host innate cellular responses to HIV infection. Since the Vpr-specific activ-

ities have been linked to such clinical manifestation of AIDS as activation of viral replication (30), suppression of host immune responses (40), and depletion of CD4⁺ T lymphocytes (47, 48), this finding could potentially provide a new approach to reducing Vpr-mediated detrimental effects in HIV-infected patients by stimulating expression of small heat shock proteins or by administering exogenous small heat shock proteins such as Hsp16.

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