Anti-Vpr Activities of Heat Shock Protein 27

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HIV-1 Vpr plays a pivotal role in viral pathogenesis and is preferentially targeted by the host immune system. In this report, we demonstrate that a small heat shock protein, HSP27, exhibits Vpr-specific antiviral activity, as its expression is specifically responsive to *vpr* gene expression and increased levels of HSP27 inhibit Vpr-induced cell cycle G2 arrest and cell killing. We further show that overexpression of *HSP27* reduces viral replication in T-lymphocytes in a Vpr-dependent manner. Mechanistically, Vpr triggers HSP27 expression through heat shock factor (HSF) 1, but inhibits prolonged expression of HSP27 under heat-shock conditions. Together, these data suggest a potential dynamic and antagonistic interaction between HIV-1 Vpr and a host cell HSP27, suggesting that HSP27 may contribute to cellular intrinsic immunity against HIV infection.

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr), a virion-associated protein with a calculated molecular weight of 12.7 kilodalton (kD), is highly conserved among HIV, simian immunodeficiency virus (SIV), and other lentiviruses (1,2). Increasing evidence suggests that Vpr plays an important role in the viral life cycle and pathogenesis. Rhesus monkeys, chimpanzees, and human subjects infected with Vpr-defective viruses show slower disease progression often accompanied by reversion of the mutated *vpr* genes back to the wild type phenotype (3–7).

Vpr displays several distinct activities in host cells and has been implicated in multiple virus-host interactions. These include induction of cell cycle G2 arrest (8) and cell killing (9). The cell cycle G2 arrest induced by Vpr is thought to suppress human immune functions by preventing T cell clonal expansion (10) and to provide an optimized cellular environment to viral replication (3). The biological role of Vpr-induced apoptosis of target cells is unclear at present, but may represent a host self-destructive mechanism to prevent viral spread or may be a viral mechanism to deplete $CD4^+$ T-cells (7,11,12).

To search for cellular proteins capable of suppressing the Vpr activities, we took a unique approach by conducting a genome-wide screening of Vpr suppressors in a fission yeast (*Schizossaccharomyces pombe*) model system (13,14). We identified a fission yeast small heat shock protein, Hsp16, that was able to specifically inhibit the Vpr activities both in fission yeast and mammalian cells (13). Further analysis showed that production of Hsp16 is responsive to *vpr* gene expression in fission yeast. In addition, there was a dynamic and mutually suppressive interaction between Vpr and Hsp16 (15). Because heat shock proteins and related regulatory factors controlling cellular heat shock responses are highly conserved among eukaryotes, we investigated the relation between Vpr and the human paralogue of Hsp16, HSP27. Consistent with earlier studies that showed that expression of *HSP27* was responsive to HIV infection (16,17), we demonstrate that HSP27 responds specifically to *vpr* gene expression during HIV infection. Furthermore, we show that artificial overexpression of *HSP27* suppresses the Vpr activities both when the latter was expressed on its own or in the context of viral infection. Thus, this responsive elevation of HSP27 expression could represent one of the host cell intrinsic antiviral mechanisms against HIV infection specifically targeted to the Vpr activities. In its turn, Vpr appears to counteract HSP27 activity by inhibiting prolonged expression of this heat shock protein.

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MATERIALS AND METHODS

Cell Growth and Gene Expression in Human Cells

$CD4^+$ H9 and CEM-SS cells were grown in RPMI 1640 medium and HEK293 cells were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Gaithersburg,

MD, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 100 unit/mL of penicillin/streptomycin. The HEK293-632 cell line is a stable zeocin-resistant cell line (18) expressing a heterodimer of the modified ecdysone receptor (VgEcR) and the retinoid X receptor (RXR). The heterodimer binds a hybrid ecdysone response element (E/GRE) only in the presence of the synthetic analogue of ecdysone, muristerone A (19,20). The transfected HEK293-632 cells expressing *vpr* and Vpr-suppressor HSP27 were selected with geneticin (750 µg/mL; Mediatech Inc., Holly Hill, FL, USA) for *vpr* cloned on the pZY1 plasmid and hygromycin (200 µg/mL) for genes cloned on the pZH1 plasmid (18). Thus, gene expression of HIV-1 *vpr* or *HSP27* was induced by muristerone A (1 µM) as described previously (18).

Cell Cycle Analysis

To measure Vpr-induced G2 arrest in *vpr*-inducible HEK293 cells, flow cytometric analysis was carried out as previously described with minor modifications (18). Briefly, approximately 1×10^6 HEK293-632 cells transfected with both pZH-*vpr* and pZY-carrying genes of interest were plated into 25 cm^2 flask and grown under selection by zeomycin (400 µg/mL), geneticin (750 µg/mL), and hygromycin (200 µg/mL) for five to seven days prior to *vpr* gene induction. 1.0 µM muristerone A was added to cell cultures to induce gene expression as previously described (18). Cells were collected in 1 mL of PBS with 5 mM EDTA, fixed by addition of 2.5 mL of cold 95% ethanol on ice 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 µg/mL) at 37°C for 30 min prior to propidium iodide (PI) staining. PI was added to a final concentration of 10 µg/mL and flow cytometric analysis was carried out after 1 h incubation on ice. The DNA content of the transfected cells was determined on a Becton-Dickinson flow cytometer by using CellQuest software.

Analysis of Apoptosis by Flow Cytometry

Vpr-induced cell death in HEK293 cells was first evaluated by trypan blue staining (Sigma, St. Louis, MO, USA). Annexin V assays were performed to further quantify the effect of HSP27 on Vpr-induced apoptosis by using the BD ApoAlert Annexin V assay kit (Cat. No. 630109, BD Biosciences, San Jose, CA, USA). Briefly, approximately 1×10^6 HEK293-632 cells transfected with both pZY1-*vpr* and pZH1 carrying *HSP27* were plated into 25 cm^2 flask and grown under selection by zeomycin (400 µg/mL), geneticin (750 µg/mL), and hygromycin (200 µg/mL) for five to seven days prior to *vpr* gene induction. 1.0 µM muristerone A was added to cell cultures to induce gene expression as previously described (18). Cells were harvested 24 to 48 h after gene induction for the BD ApoAlert Annexin V assay or trypan blue staining. For the Annexin V assay, cells were analyzed on a Becton-Dickenson flow cytometer and Annexin V-FITC fluorescent signals were detected and analyzed by software CellQuest.

Analysis of Cell Death by Trypan Blue Staining

Cells were treated with tripsin, resuspended in DMEM, and mixed 1:1 with trypan blue solution. Blue cells were counted under microscope; over 1,000 cells per field were counted, and three fields per each sample were analyzed. The experiments were repeated at least three times.

Evaluation of the Effects of HSP27 on the Vpr Activities During Viral Infection

Vpr-positive [Vpr(+)] and Vpr-negative [Vpr(-)] HIV-1, which were pseudotyped with an VSV-G envelope, were used to infect HEK293 cells carrying muristerone A-inducible *HSP27* expressing vector. The Vpr-negative construct had a single base mutation at the start codon of the *vpr* open reading frame, which changes ATG to GTG (21). For pseudotyping, infectious pNLHX clone (22) was cotransfected into HEK293 cells together with the VSV-G-expressing plasmid pHEF-VSVG obtained from the NIH AIDS Reagent Program (23). Cells (3×10^6) were infected with the VSV-G-pseudotyped Vpr(+) or Vpr(–) HIV-1 vector. The samples were normalized by RT activity to 3.5×10^7 cpm/mL. To determine the effect of HSP27 on Vpr-induced G2 arrest, cell cycle profile of infected cells was analyzed by flow cytometric analysis 48 h after infection. To evaluate potential suppressive effect of HSP27 on cell killing caused by HIV infection in $CD4^+$ T-lymphocytes, $CD4^+$ H9 or CEM-SS cells that stably express a plasmid control and *HSP27* were established. 3×10^6 to 5×10^6 of these cells were either mock infected or infected with 2000 $TCID_{50}$ of HIV-1_{LAI}. Equal infection of the cells was further verified by measuring viral RNA levels 24 h after viral infection using the Roche Monitor assay following the manufacturer's instructions. Cytotoxicity caused by HIV-1 infection was measured by staining dead cells with trypan blue or with the MTT assay following the manufacturer's instruction (Boehringer Mannheim, Indianapolis, IN, USA). Viral replication was determined in H9 and CEM-SS cells by p24 antigen levels using a commercially available HIVAG-1 polyclonal antigen kit (Abbott Laboratories, Abbott Park, IL, USA). Both H9 and CEM-SS cells are $CD4^+$ and were derived from human T-lymphocytes (24,25).

Other Molecular and Cellular Techniques

All mammalian transfections were conducted by electroporation on a Bio-Rad GENE Pulser II following the manufacturer's protocols. Five to 10 µg of DNA were normally used per transfection. Induction of cellular heat shock responses were conducted as previously described (26). Briefly, approximately 1×10^6 HEK293-632 cells transfected with both pZY1-*vpr* and pZH1-HSP27 *HSP27* were first grown as mentioned above to fully express Vpr and HSP27. Heat treatment was done by growing

these cells at 43°C for 30 min. and then transfer them back to 37°C for testing at indicated time intervals. For Western blot analysis, cells were harvested and rinsed with ice-cold HEPES-buffered saline (pH 7.0), then lysed in an ice-cold cell lysis buffer: 20 mM Tris-HCl, pH7.6, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, 5 μM Trichostatin A, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM NaF, and protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). Cellular lysates were prepared and the protein concentration was determined using the Pierce protein assay kit. For immunoblotting, an aliquot of total lysate proteins (30 or 50 μg) in 2× SDS-PAGE sample buffer (1:1 v/v), was electrophoresed and transferred to a nitrocellulose membranes. The membranes were incubated with Tris-buffered saline-tween 20 (TBST) and 5% skim milk for 1 h, and were then incubated with appropriate primary antibody in TBST (pH 7.5) and 2% skim milk overnight. After washing, the membrane was incubated with secondary antibody in TBST buffer for 1 h. Protein bands were visualized by an ECL detection system. The Student's *t*-test was used to determine the statistical significance of differences between experimental groups as indicated by *P*-values; $P < 0.05$ was considered significant. pSM2c plasmid carrying a nucleotide sequence (5′-TGCTGTTGACAGTGAGCGCCCACAGAGATACACAGATATATAGTGAAGCCACAGATGTATATATCTGTGTATCTCTGTGGATGCCTACTGCCTCGGA-3′) that encodes a short hairpin RNA sequence (shRNA) against human HSF1 is commercially available from Open Biosystems, Inc. (Cat. No. RHS1764-9689169, Huntsville, AL, USA).

RESULTS

Endogenous HSP27 is Responsive to *vpr* Gene Expression

We previously identified fission yeast (*S. pombe*) Hsp16 as a potent Vpr suppressor (13). Subsequent analysis of Hsp16 expression in *S. pombe* demonstrated that Vpr elicited Hsp16 production (15). Comparison of protein sequences of fission yeast Hsp16 and human HSP27 suggests that HSP27 might be a functional paralogue of Hsp16 because they both share the same motif of the HSP20/α crystalline family (27) and anti-HSP27 antibodies weakly cross-reacted with the Hsp16 (results not shown). To determine whether Vpr elicits its activation of HSP27, we tested expression of *HSP27* in a HEK293-632 cell line (DL3) that carries an inducible *vpr* gene (13,18). Under the normal growth conditions, HSP27 protein levels in HEK293 cells are typically very low or undetectable (Figure 1A, lane 1). When *vpr* gene expression was induced with muristerone A, transient elevation of HSP27 was detected by Western blot analysis. A 4.8 ± 0.2-fold increase of HSP27 was initially seen 24 h after *vpr* gene induction; the maximum level of HSP27, which was 12.1 ± 0.1-fold higher than the basal level, was detected and lasted for about 6 h (Figure 1B). However, gradual decline of HSP27 was observed thereafter, although *HSP27* expression was maintained at a level that was 4.0-fold higher than the basal level for at least 48 h. The responsive elevation of HSP27 was observed only when *vpr* gene was expressed, as the same cells without gene induction did not show elevation of HSP27 overtime (Figures 1A,B). Treatment of HEK293 cells with muristerone A did not significantly induce HSP27 increase (data not shown). Therefore, Vpr induces transient expression of HSP27.

We next tested whether the expression of *HSP27* is Vpr-responsive during HIV-1 infection. The Vpr(+) or Vpr(–) viruses were used to infect CEM-SS and H9 $CD4^+$ T-lymphocytes. A rapid increase of HSP27 was seen in both cell lines at 3 h after infection with either virus (Figure 1C; only CEM-SS data are shown). However, the level of *HSP27* expression in cells infected with Vpr(–) virus did not increase beyond the initial level observed at 3 h p.i., whereas in cells infected with Vpr(+) virus HSP27 levels reached maximum at 5 h post-infection (Figure 1C). Similar to what we observed in Vpr-transfected HEK293 cells, the HSP27 level in cells infected with Vpr(+) virus dropped after 7 h of viral infection and was sustained at a level that was slightly lower than the level observed at 3 h after infection. Therefore, the activation of HSP27 by HIV-1 occurs both in a Vpr-dependent and Vpr-independent fashion, with Vpr being responsible for HSP27 increase between 3 and 7 h after infection. Vpr-independent activation of HSP27 likely reflects cellular responses to other HIV-1 proteins.

HSP27 Reverses Vpr-induced G2 Arrest

To test the biological significance of Vpr-dependent elevation of HSP27, we established three cell lines stably expressing HSP27: HEK293-HSP27, CEM-SS-HSP27, and H9-HSP27. Two Vpr activities, which include cell cycle G2 arrest and cell killing, were tested in these cells, as these activities have been implicated as factors in HIV-1 replication and pathogenesis (3,28,29).

To test the effect of HSP27 on Vpr-induced G2 arrest, we first used the muristerone A-inducible *vpr* gene expression system in the HEK293-632 cell line (13,18). As shown in Figure 2A, addition of muristerone A to control cell line HEK293-632 not carrying *vpr* did not affect cell cycle profile 72 h after gene induction (G2/G1 ratio of 1.10 ± 0.70, top row). In contrast, a significant increase in G2/G1 ratio (2.31 ± 0.60) was observed when the *vpr* gene was expressed, indicating accumulation of G2-arrested cells in *vpr*-expressing cultures (13, Figure 2A, second row). A stable and constitutive expression of *HSP27* did not affect cell cycle profile (data not shown) but reversed Vpr-induced G2 arrest (Figure 2A, bottom row). Western blot analysis showed that both Vpr and HSP27 were properly produced, indicating a specific suppression of Vpr-induced G2 arrest by HSP27 (Figure 2B).

To test whether the suppressive effects of HSP27 on Vpr-induced G2 arrest

observed in HEK293 cells also apply to viral infection, we used VSV-G pseudotyped HIV-1 constructs to infect HEK293-HSP27 and control, HEK293-pZY1, cells (22,23). Isogenic Vpr(+) and Vpr(–) viruses were used for infection. As shown in Figure 2C (top middle panel), infection of control cells with Vpr(+) virus caused a dramatic shift of G2/G1 ratio to 1.98, indicating a cell cycle G2 arrest due to viral infection. In contrast, infection of HEK293-pZY1 cells with Vpr(–) virus did not affect the cell cycle profile (top right panel) indicating that the G2 shift was caused specifically by Vpr. Consistent with the effect of HSP27 on Vpr expressed alone, overproduction of HSP27 (bottom middle panel) completely reversed the cell cycle profile, with a relative G2/G1 ratio of 0.96. It should be noted here that this experiment was carried out together with previously published analysis of the effect on Vpr activities of fission yeast Hsp16 (13), therefore, the control panels (Figure 2C, upper row) are the same as in Figure 1C of that manuscript. It appears that the suppressive effect of HSP27 on Vpr-induced G2 arrest is stronger than that of Hsp16 (13). Together, these results indicate that high level expression of HSP27 suppresses cell cycle G2 arrest induced by Vpr.

HSP27 Blocks Vpr-induced Cell Death and Apoptosis

Prolonged cell cycle arrest leads to cell death. The potential effect of HSP27 overproduction on Vpr-induced cell death was first measured by trypan blue assay in HEK293-632 cells 72 h after *vpr* gene induction (Figure 3A). A low basal level of cell death (11–13%) was observed in all of the cell cultures without *vpr* gene induction (Figure 3A, left panels). A similar background level of cell death (14 ± 5%) also was observed in the HEK293-632 control cells treated with muristerone A (top right panel). In contrast, an increase in dead cell-specific staining (43 ± 11%) was found in the *vpr*-expressing cell culture (Figure 3A, middle row, right panel). However, cell death

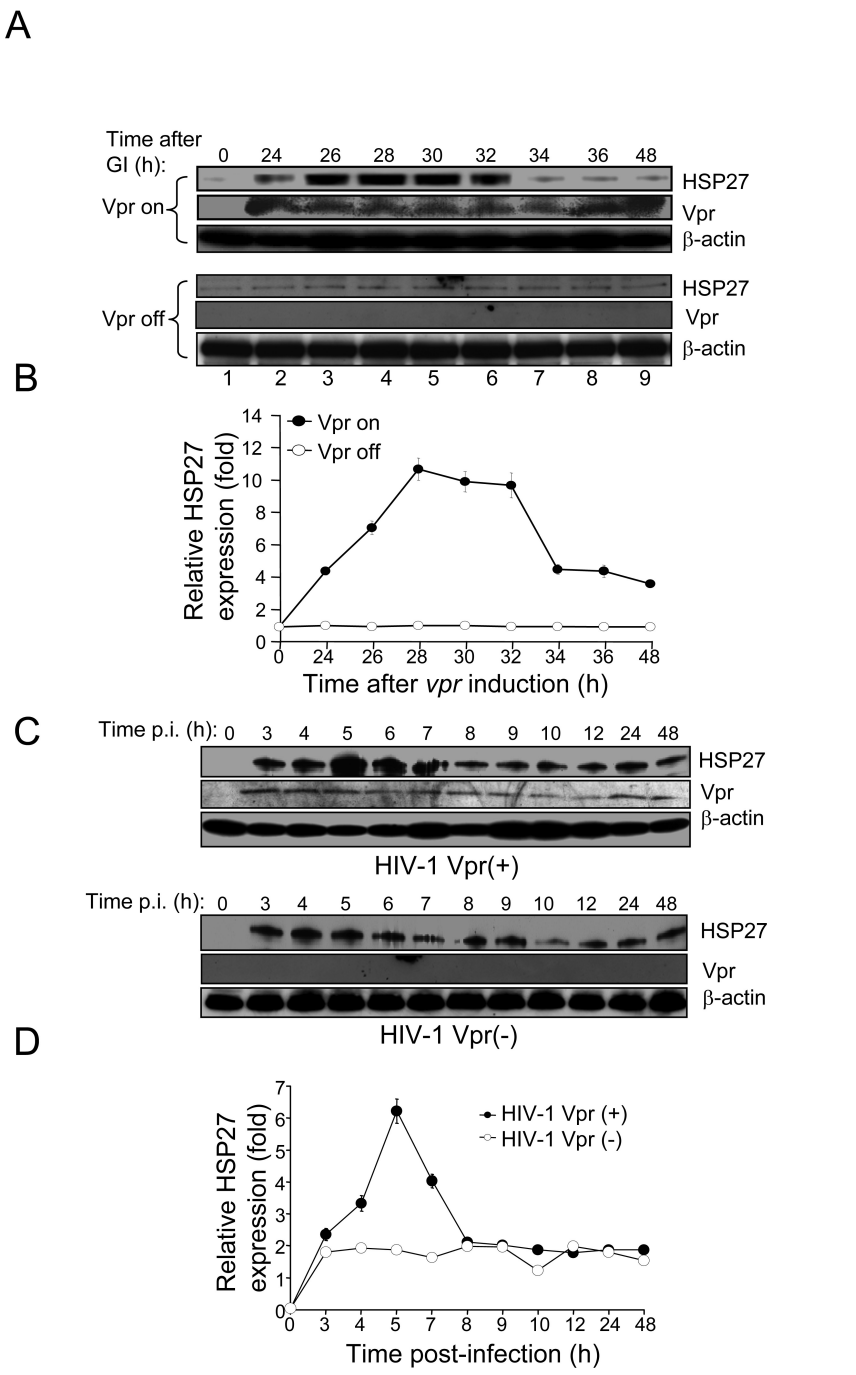

Figure 1. Expression of endogenous HSP27 is responsive to HIV-1 infection in a Vpr-dependent manner. (A) Expression of *vpr* triggers an increase of HSP27 production in HEK293 cells. The *vpr* gene expression was induced by muristerone A as described (18). Following indicated times after gene induction (GI), HSP27 protein levels were determined by Western blot analysis. (B) Quantification of results in panel A by densitometry. Results show mean ± SD calculated from three independent experiments. (C) Analysis of HSP27 production in HIV-infected CEM-SS T-lymphocytes. The level of HSP27 expression was examined at indicated time intervals post infection (p.i.) with HIV-1. Top panels show results with Vpr(+) virus; bottom panels – with Vpr(–) HIV-1. (D) Quantification of results in panel C by densitometry. Results show mean ± SD calculated from three independent experi-

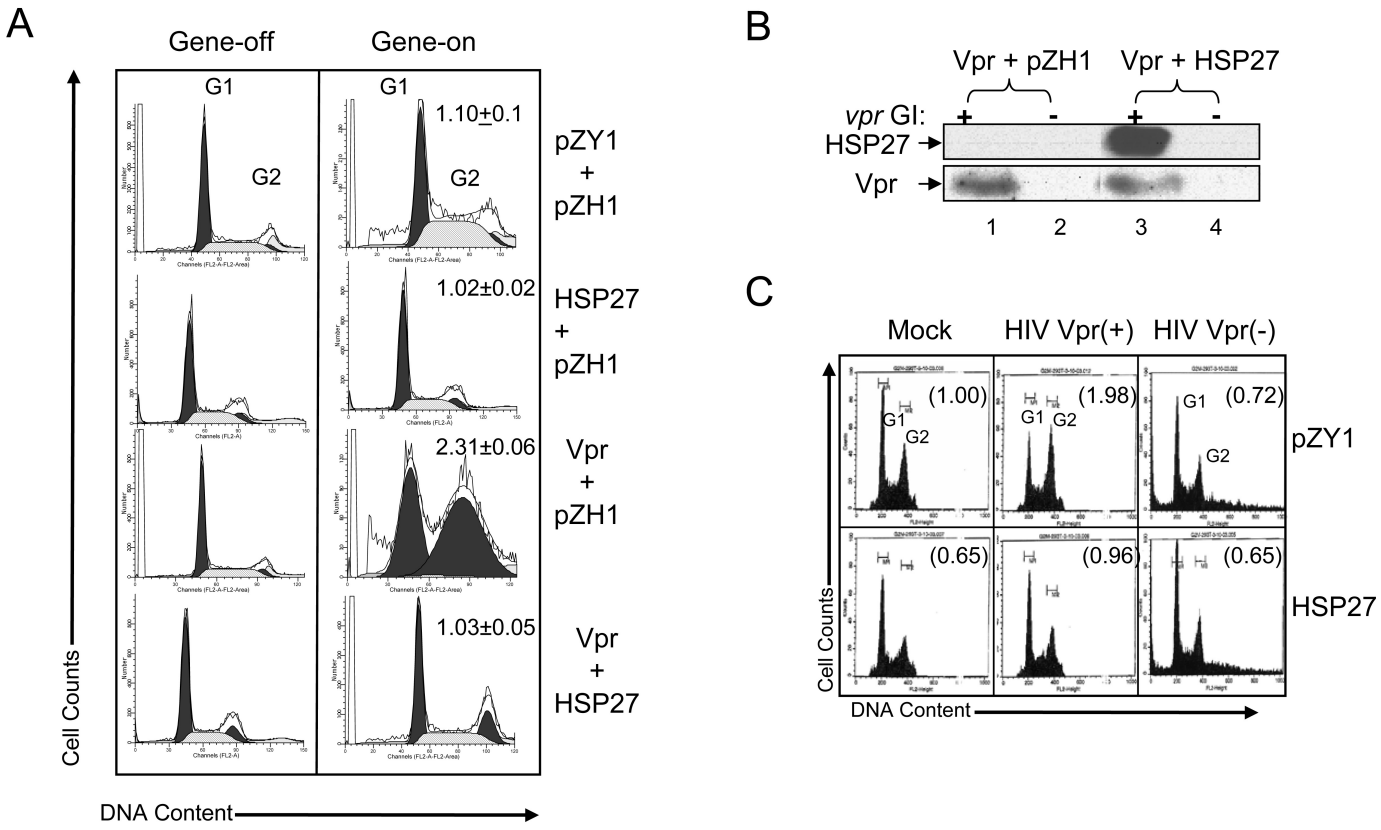

Figure 2. Overexpression of HSP27 suppresses Vpr-induced cell cycle G2 arrest. (A) Suppression of Vpr-induced G2 arrest by HSP27 in Vpr-transfected HEK293-632 cells. Both the *vpr* and *HSP27* genes were induced by muristerone A as described previously (18) through pZY1-*vpr* or pZH1-*HSP27* plasmids, respectively. The extent of Vpr-induced G2 arrest, measured by flow cytometric analysis, was quantified 72 h after gene induction, by the relative G2 to G1 ratio between gene-repressing and gene-expressing cells. A relative G2/G1 ratio close to one indicates no significant difference between the *gene*-on and *gene*-off cultures. A relative ratio > 1 suggests increased proportion of G2 cells in the *gene*-on culture (18). A representative experiment out of three performed is shown; the following *P* values have been calculated: (pZY + pZH) vs. (Vpr + pZH), $P < 0.05$; (Vpr + HSP27) vs. (Vpr + pZH), $P < 0.05$; (pZY + pZH) vs. (Vpr + HSP27), $P = 0.31$. (B) Western blot analysis of Vpr and HSP27 on an aliquot of cells collected for flow cytometric analysis in panel A. (C) VSV-G pseudotyped Vpr(+) or Vpr(–) HIV-1_{NLHX} (22) was used to infect *HSP27*-expressing cells. Cell cycle distribution was measured by flow cytometry 48 h after viral infection. Note that this experiment was performed together with analysis of Hsp16 activity, which was published previously (13); therefore, the control panels are the same as shown in that paper.

was reduced to near background levels (18 ± 3%) when HSP27 was co-expressed with *vpr* (Figure 3A, bottom row, right panel).

We next tested the potential suppressive effect of HSP27 on Vpr-induced apoptosis using flow cytometric analysis of Annexin V staining. Annexin V-positive cells typically represent early (Figure 3B, bottom right quadrant) and late (Figure 3B, upper right quadrant) apoptotic cells (30). As shown in Figure 3B, un-induced cells showed background level of apoptosis ranging from 0.20% to 0.32% for early apoptosis and from 0.74% to 2.46% for late apoptosis. Gene induction of *HSP27* alone did not alter the number of apoptotic cells (data not shown). Similarly, addition of muristerone A to control cells had no effect on apoptosis (Figure 3B, top right panel). Marked increases were found both in the early and late apoptosis (18.26% and 11.45%, respectively) when *vpr* gene expression was turned on by adding muristerone A to HEK 293-632 cells. Consistent with the results shown in Figure 3A, stable expression of HSP27 partially reduced the number of cells in early and late apoptosis, to 6.51% and 5.60%, respectively (Figure 3B, bottom right panel).

Because HEK293 cells are not a natural host for HIV infection, we next tested the suppressive effect of HSP27 on Vpr-induced cell killing in H9-HSP27 and CEM-SS-HSP27 cell lines stably expressing the *HSP27* gene, or control (vector-transfected) H9 and CEM-SS. These cells were either mock-infected or infected with HIV-1_{LAI} (Figure 3C – only H9 data are shown). Equal infection of control and *HSP27*-transfected cells was con-

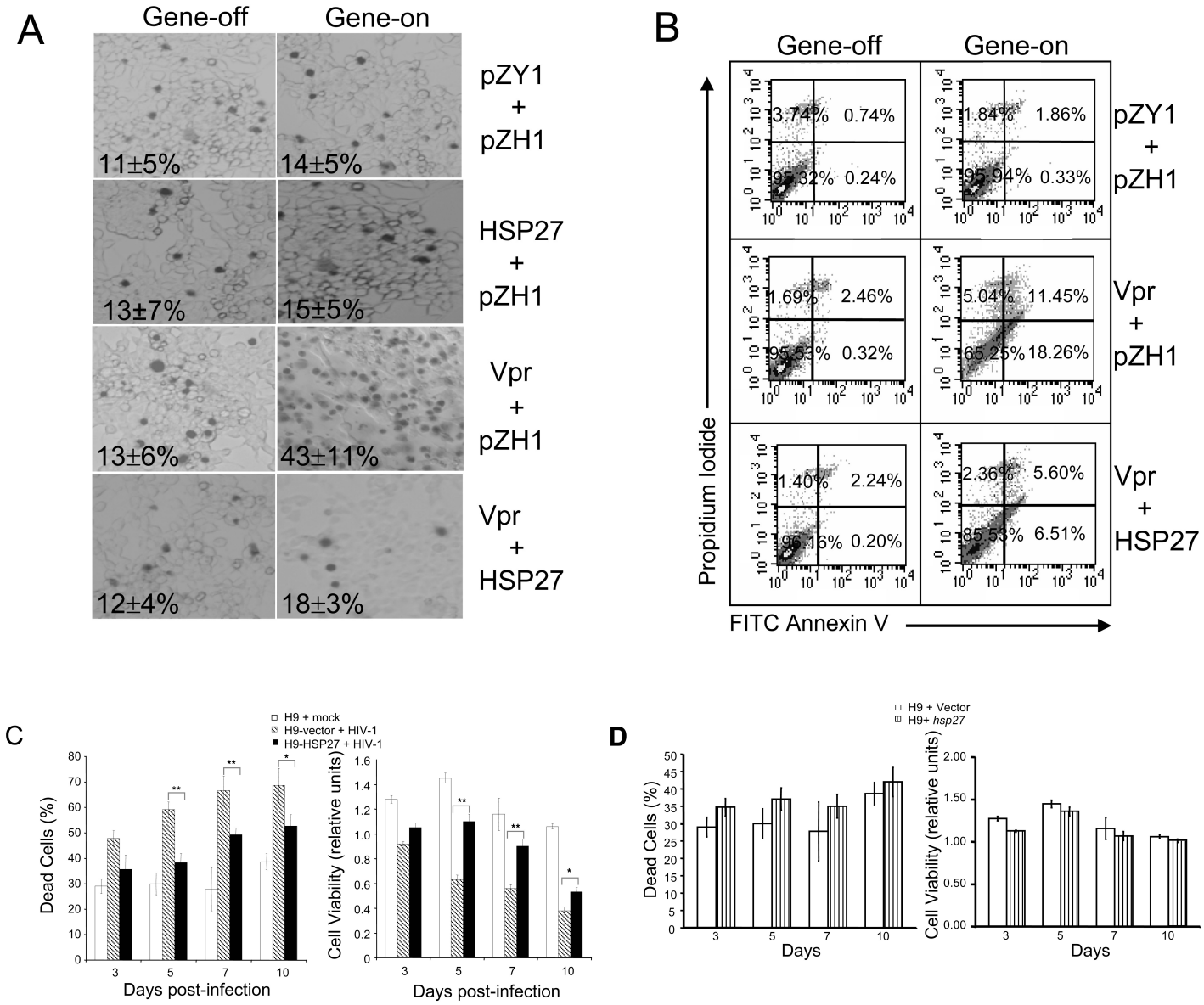

Figure 3. Overexpression of HSP27 suppresses Vpr-induced cell death and apoptosis. (A) Suppression of Vpr-induced cell death by HSP27 in *vpr*-transfected HEK293-632 cells. Vpr and HSP27 expression was induced by muristerone A. Dead cells were detected by trypan blue straining 72 h after gene induction. (B) Anti-apoptotic activity of HSP27 in HEK293-632 cells. Apoptosis was detected by Annexin V assay using a BD ApoAlert Annexin V kit. Annexin V-positive cells that appear in the low right quadrant typically represent early apoptosis and cells in late apoptosis are found in the upper right quadrant (30). (C) HSP27 overexpression reduces cytopathic effect of HIV-1 infection. Dead cells (trypan blue-positive) were counted in HIV-infected H9 T-lymphocytes by trypan blue assay (left panel); cell viability was determined by an MTT assay (right panel). H9 cells were either mock-infected (H9 + mock), infected with HIV-1_{LAI} without overexpressed *HSP27* (H9-vector + HIV-1) or infected with HIV-1_{LAI} with overexpressed *HSP27* (H9-HSP27 + HIV-1). Cell death and cell viability of the infected cultures were examined three, five, seven, and ten days after infection. Percentage of cell death induced by HIV-1 infection was quantified by counting the number of blue cells over total cell population. Cell viability was quantified by measuring optical density at 630 nm using a commercial MTT assay kit. Average and standard errors represent a total of three independent experiments. *, statistical significance at the level of $P < 0.001$; **, statistical significance at the level of $P < 0.0001$. (D) Overexpression of HSP27 does not have an effect on cell viability. Cell death and cell viability were examined in un-infected H9 cells as in panel C.

firmed by determining the viral RNA levels produced by the cells 24 h after infection. The viral RNA ranged from $10^{4.8}$ to $10^{4.9}$ copies/mL in both HSP27-transfected and control cells. Cell death caused by HIV-1 was evaluated three, five, seven, and ten days after viral infection by staining with trypan blue (Figure 3C, left panel) or by measuring cell viability using the MTT assay (Figure 3C, right panel). The background cell death ranged from 27.8 ± 8.5% to 38.7 ± 3.2% in mock-infected cells. A steadily increasing percentage of dead cells (47.8 ± 3.7% to 68.7 ± 6.7%) was observed from day three to day ten after viral infection in HIV-infected cultures carrying the control vector. In contrast, a 12.2% reduction in cell death was seen in the *HSP27*-expressing cells three days after viral infection, and 20.8% to 16.0% reduction was observed from five to ten days after viral infection. A similar suppression of cell death by HSP27 was also found when the MTT assay was used to determine cell viability (Figure 3C, right panel). Cell viability decreased from 0.9 ± 0.1% to 0.4 ± 0.0% from day three to day ten after viral infection of control cells (Figure 3C, right panel). At five to ten days after infection, 39.5% to 74.6% more viable cells were recovered in *HSP27*-expressing cultures, even though there was no significant increase of cell viability at day three. Western blot analysis indicated that HSP27 was expressed at high level in both cell lines carrying HSP27 vector, whereas normally these cells express very low or undetectable levels of HSP27 (data not shown). Importantly, the effect of HSP27 was specifically targeted at Vpr-induced cell death, as over-expression of HSP27 in uninfected cells did not alter cell viability (Figure 3D). Thus, overproduction of HSP27 confers a partial protective effect against Vpr-induced cell death and apoptosis.

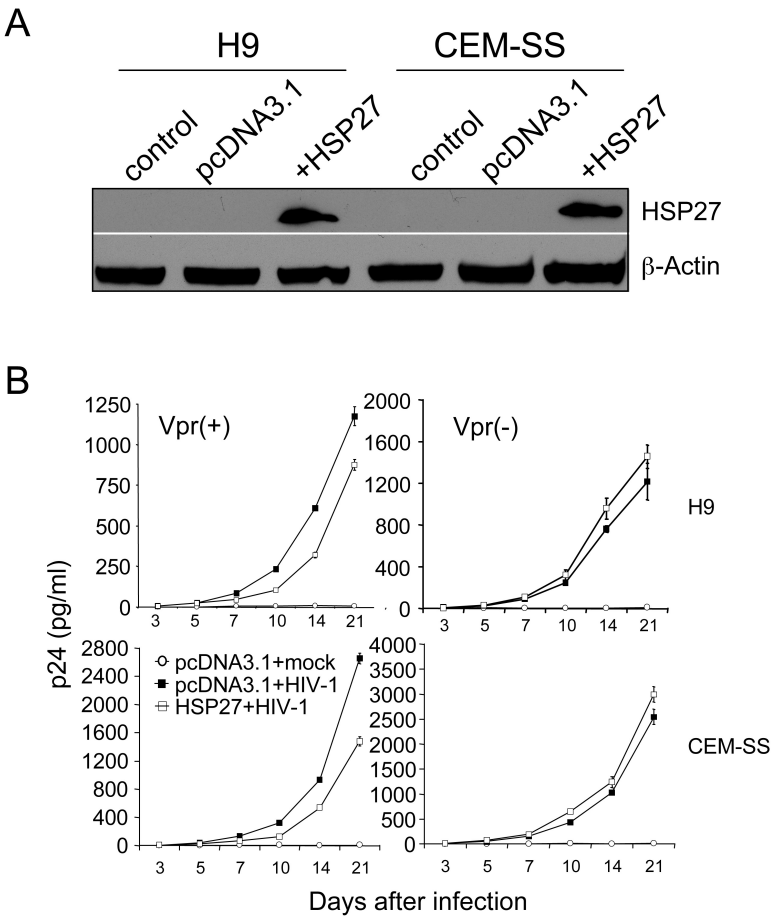

Figure 4. HSP27 inhibits viral replication in CEM-SS and H9 T-lymphocytes in a Vpr-dependent manner. (A) HSP27 expression analysis in stably transfected cells by Western blot. Ctr – mock-transfected cells; lanes 1–3: H9 cells; lanes 4-6: CEM-SS cells; pcDNA3.1 – cells transfected with an empty vector; + HSP27 – cells transfected with *HSP27*-expressing plasmid. (B) Stable expression of *HSP27* reduces viral replication in CD4 + H9 and CEM-SS cells in a Vpr-dependent manner. Viral replication was determined by measuring p24 antigen in culture supernatant at indicated time. *, indicates statistical significance (at the level of $P < 0.01$) between HIV-1_{NLHX}-infected cells with or without overexpression of HSP27.

HSP27 Inhibits Viral Replication in a Vpr-dependent Manner

Vpr activities have been implicated as a positive factor in HIV-1 replication (3,28) and contribute to 2.0- to 4.0-fold increase of viral replication in proliferating T lymphocytes of Vpr(+) vs. Vpr(–) HIV-1 (3,13,31). Because HSP27 counteracts Vpr activities, we reasoned that HSP27 might inhibit HIV-1 replication. To test this possibility, we used $CD4^+$ H9 and CEM-SS cell lines stably producing high levels of HSP27 (Figure 4A). These cells were infected with two matched pNLHX viral clones (16) that differ only in the *vpr* gene. We then measured the p24 antigen production in culture supernatants over a period of 21 days after infection. As shown in Figure 4B (left panels), a moderate but consistent reduction of HIV-1 viral replication was observed in H9 cells expressing *HSP27*. For example, levels of p24 antigen steadily increased in HIV-infected cells expressing the vector control from day three to day 21 of HIV-1 infection indicating successful viral infection (Figure 4B, top left panel). However, a 1.3- to 2.3-fold reduction in p24 antigen levels was detected in HIV-infected cells expressing *HSP27*

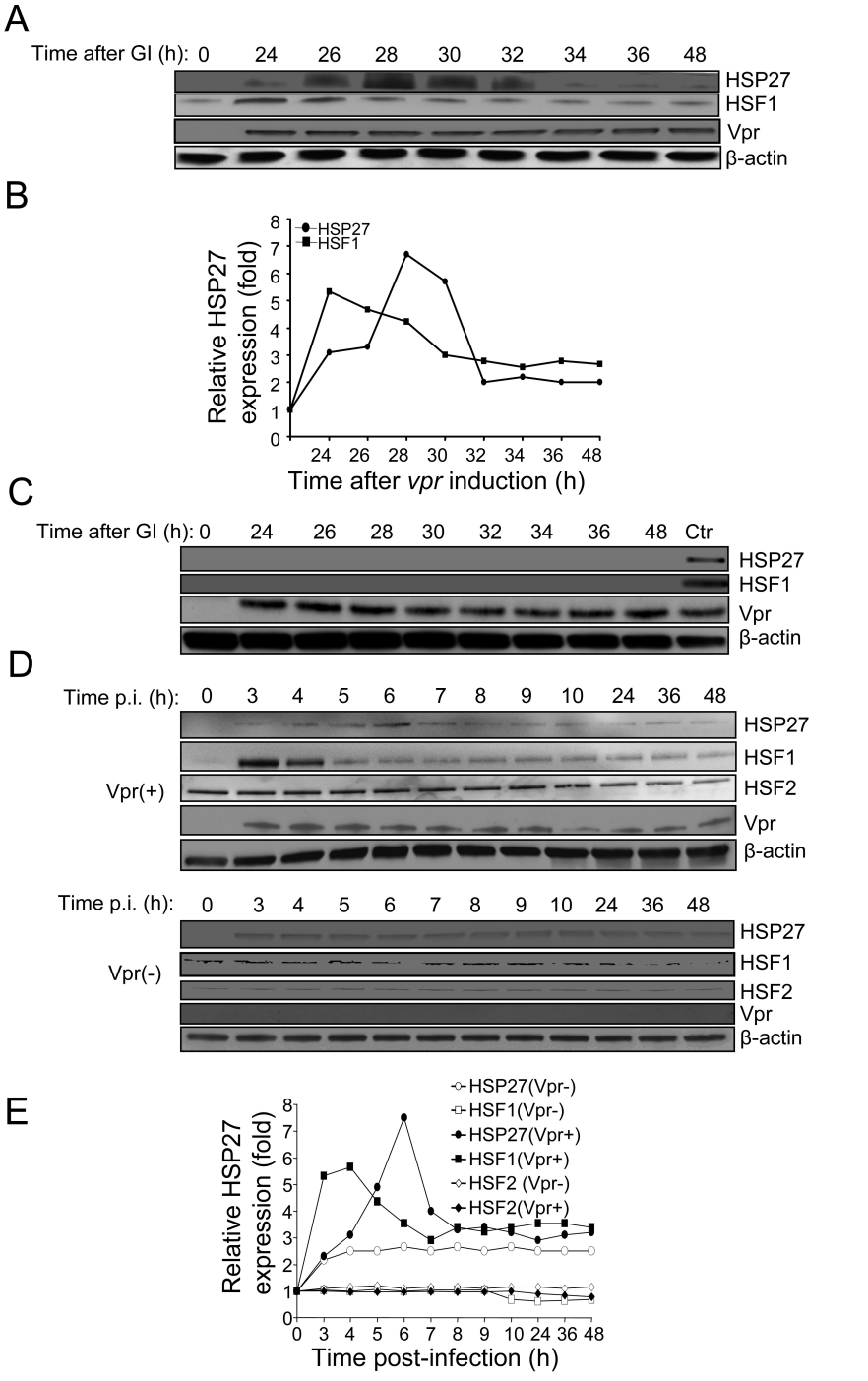

Figure 5. Activation of HSP27 is mediated through HSF1. (A) Analysis of *Vpr*, *HSF1* and *HSP27* expression in HEK293 cells carrying muristerone A inducible *Vpr* by Western blotting at various time points after *vpr* gene induction (GI). (B) Quantification of Western blot analysis in panel A by densitometry. (C) *HSF1* expression was knocked-down using shRNA, and expression of indicated proteins was analyzed after *vpr* gene induction (GI). Cell extracts without anti-HSF1 shRNA were used as the positive control (Ctr) to show production of HSF1 and HSP27. (D) Analysis of CEM-SS cells infected with Vpr(+) or Vpr(–) HIV-1. Analysis by Western blotting was performed at indicated times after infection. (E) Results in panel D were quantified by densitometry.

from day 10 to day 21 after viral infection. No detectable p24 antigen was observed in mock-infected cells over the entire experimental period. To ensure that the observed suppressive effect of HSP27 was not cell line-specific, we also examined CEM-SS, another $CD4^+$ cell line derived from T lymphocytes. A similar suppressive effect on viral replication (1.8- to 2.6-fold reduction) was observed in CEM-SS cells stably expressing *HSP27* gene (Figure 4B, bottom left panel). Importantly, no effect of HSP27 on replication of Vpr(–) HIV-1 was observed (Figure 4B, right panels), suggesting that the anti-HIV activity of HSP27 is Vpr-specific. The modest effect of HSP27 on viral replication in this system likely reflects a relatively minor contribution of Vpr to HIV-1 replication in T cell lines.

Activation of HSP27 is Mediated Through HSF1 and is Counteracted by Vpr

In a yeast study, we found that gene expression of fission yeast *Hsp16* is responsive to *vpr* gene expression and that Vpr-dependent Hsp16 elevation is mediated through a heat shock factor (HSF)-dependent mechanism (15). HSF1 and HSF2 are upstream regulators of HSP-mediated stress responses (32). We tested whether Vpr effects in mammalian cells involved an HSF-dependent mechanism similar to that observed in yeast cells. Experiments were performed in HEK293-632 cell line carrying inducible *vpr* as described in Figure 1A. Temporal changes in human HSF and HSP27 expression were determined. Consistent with results in Figure 1A, HSP27 was transiently elevated from 26 to 32 h after *vpr* gene induction (Figure 5A, upper row). Elevation of HSF1 in response to *vpr* gene expression was observed earlier than HSP27, the maximum level of HSF1 was detected at 24 h after *vpr* gene induction, whereas HSP27 reached its maximum level 4 h later (Figure 5B).

To make certain that HSF1 activation by Vpr is critical for HSP27 elevation, we suppressed *HSF1* gene expression using a short hairpin RNA (shRNA) and car-

ried out the same experiment as described in Figure 5A. Depletion of HSF1 completely abolished HSP27 elevation after *vpr* gene induction (Figure 5C).

We next analyzed the kinetics of HSF and HSP27 expression after HIV-1 infection. Consistent with results in Figure 1C, HSP27 protein was initially detected at about 3 h after infection, staying constant in cells infected with Vpr-deficient virus, while reaching maximum level at 6 h and then decreasing to the 3-h level in cells infected with Vpr(+) virus (Figure 5D). Only HSF1, but not HSF2, was elevated in response to Vpr(+) HIV-1 infection. The *HSF1* gene expression appeared to precede elevation of HSP27, as its protein level climbed from barely detectable to maximum level within 3–4 h after viral infection, which is approximately 3 h ahead of the time when maximum level of HSP27 was observed (Figure 5E). In contrast, no apparent elevation of HSF1 was seen in CEM-SS cells infected with Vpr(–) virus (Figure 5E). Vpr-specific elevation of HSF1 and HSP27 appears to happen earlier in viral infection than in response to *vpr* gene induction. This difference may be due to rapid release of Vpr protein from the viral particles during viral infection relative to extended time needed for *vpr* gene expression from a plasmid. Taken together, these results suggest that HSF1 activation is Vpr-dependent and that HSP27 activation in HIV-infected cells is regulated by HSF1 but not HSF2.

The elevation of HSP27 appears to be transient as it eventually declines (Figures 1B,D and 5B,E). To test whether sustained Vpr expression reverses elevation of HSP27 in mammalian cells, HSP27 protein level was artificially elevated by treating cells at 43°C for 30 min in the presence or absence of Vpr. Consistent with the property of a heat shock protein, a rapid increase (10.8 ± 0.5-fold above background) of HSP27 was observed after 4 h of heat treatment (Figure 6A, upper panel). However, the HSP27 protein level returned back to background at approximately 48 h. When the same heat treatment was conducted with the *vpr*-expressing cells, a similar transient expression profile of HSP27 was also seen (Figure 6A, bottom panels). However, a 2.0 to 4.4-fold decrease of HSP27 was detected over time in cells expressing *vpr* in comparison with cells without Vpr (Figure 6B). These data suggest that Vpr counteracts the elevation of HSP27 triggered by heat shock. A similar effect was observed with Hsp16 in yeast cells (15).

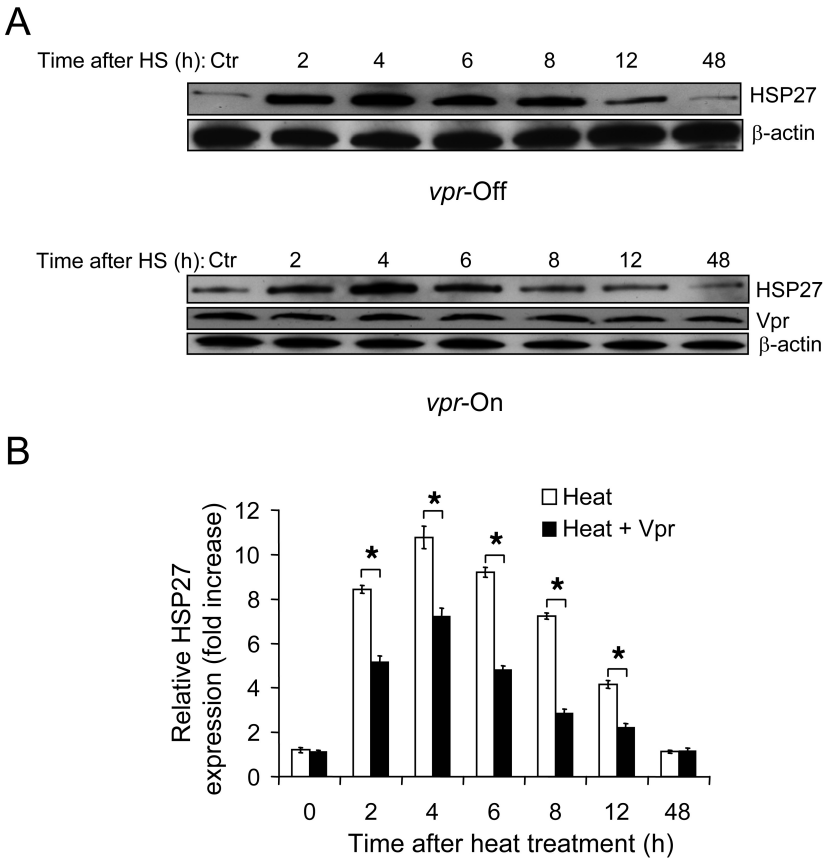

Figure 6. Vpr reduces elevation of HSP27 induced by heat treatment. (A) Western blot analysis of HEK293-262 cells carrying muristerone A-inducible *vpr*. Cells were treated (*vpr*-on) or not (*vpr*-off) with muristerone A, then treated at 43°C for 30 min and analyzed by Western blotting at indicated time intervals after heat shock (HS). (B) Quantification of Western blot analyses in panel A by densitometry; * statistical significance at the level of $P < 0.05$.

DISCUSSION

In this report, we demonstrate that several pathogenic activities of Vpr are counteracted by a small heat shock protein HSP27. Specifically, sustained and high level expression of HSP27 inhibits Vpr-induced cell cycle G2 arrest, cell killing, and suppresses HIV replication in a Vpr-dependent manner. We further demonstrate that HSP27 production is responsive to *vpr* gene expression either alone or in the context of HIV infection. Interestingly, while Vpr triggers expression of *HSP27*, it also reduces the level of HSP27 expression. Both these effects might involve HSF1, an upstream signaling molecule regulating stress responses. Although the mechanistic details of Vpr-dependent induction and restriction of *HSF1* expression remain to be further delineated, our finding that *vpr* gene expression elicits HSF1 – but not HSF2-mediated HSP27 activation suggests that Vpr triggers a cellular stress response

that may specifically involve HSF1-mediated cellular events. Indeed, earlier studies have shown that HSF1 and HSF2 are characteristically different in their ways to regulate stress responses (32). HSF1 is a rapid responder to heat-related stresses, whereas HSF2 is an active responder to hemin treatment but refractory to heat stress (32–34). Therefore, it is reasonable to assume that *vpr* gene expression may activate a cellular stress response that is similar to heat shock response. Because HSF1 modulates heat shock protein production by transcriptional regulation (32–34), it is likely that the HSP27 response to Vpr is regulated at the transcriptional level. This notion is certainly supported by our studies in fission yeast showing that activation of Hsp16 occurs at the transcriptional level and Vpr triggers a cellular response that mimics cellular heat shock response (13,15t).

Similar to what we have observed in fission yeast, Vpr also appears to counteract the antiviral response of HSP27, possibly through transcriptional regulation. This premise is supported by the finding that responsive natural elevation of HSP27 is not sufficient to restrict the Vpr activities, whereas overexpression of *HSP27* under the control of an exogenous and constitutive promoter overrides the suppressive effect of Vpr (Figure 2, Figure 3). The transcriptional mechanism of HSP27 suppression was further implicated by our observation that Vpr prevents the activation of HSP27 induced by heat treatment (Figure 6B). Moreover, transcriptional inhibition of *HSP27* by HIV infection has been demonstrated previously (16,17), providing additional support to the idea that Vpr antagonizes HSP27 at the transcriptional level. Obviously, a direct transcriptional profiling of the effects of Vpr on *HSP27* gene expression is required to substantiate this possibility.

Earlier studies of *HSP27* gene expression in HIV-infected $CD4^+$ T-lymphocytes indicated an active interplay between production of HSP27 and HIV infection (16,17). For example, *HSP27* mRNA transcription appeared as early as 3–8 h following HIV infection. However, the *HSP27* mRNA transcript was significantly down-regulated by 24 h, concomitant with the first appearance of full-length genomic HIV-1 mRNA (16). We report here that this phenomenon is Vpr-specific. In addition, there appears to be a dynamic interaction between the *vpr* gene expression and activation of HSP27. For example, expression of *vpr* either alone (Figure 1A) or in the context of HIV infection (Figure 1C) elicits a transient increase of the HSP27 protein level, suggesting that Vpr is likely responsible for the increased production of HSP27 reported previously (16).

Previously, we reported that another heat-shock protein, HSP70, has activities similar to those described here for HSP27: it suppresses Vpr-induced cell cycle arrest and apoptosis, and inhibits HIV replication (35,36). Although these two chaperones function through different mechanisms (HSP70/HSC70 is an ATP-dependent chaperone, whereas activity of HSP27 is ATP-independent but requires oligomerization of the protein), their activities may overlap. For example, both chaperones have been shown to promote the degradation of proteins through ubiquitin-proteasome pathway or inhibit apoptosis (37). The observed anti-Vpr activities of the heat-shock proteins may also involve stimulation of the proteasomal degradation of either Vpr or proteins that are required for the Vpr activities, a possibility that is currently under investigation. Despite these similarities, there are also clear differences in the mode of action of these two families of heat shock proteins. HSP70 binds Vpr and inhibits all Vpr activities, including effects on cell cycle, apoptosis, and nuclear import (35,36). In contrast, we could not detect Vpr-HSP27 interaction, even when both proteins were overexpressed (result not shown). On a functional level, HSP27 reduces Vpr-specific cell cycle arrest and apoptosis, but has only a small effect on Vpr-mediated nuclear import (35,36).

HSP27 activities described in this report are consistent with those of an intrinsic immunity factor targeting Vpr. Indeed, HSP27 is produced in response to Vpr, its expression is modulated by Vpr, it can block Vpr-specific activities, and it suppresses HIV replication in a Vpr-specific fashion. Given that Vpr-mediated cell cycle arrest is a highly conserved function of all primate lentiviruses (38,39), Vpr appears to be a good target for innate antiviral factors. The evidence presented here, along with results of our other studies in human and fission yeast cells (13,35), indicate that overexpression of heat shock proteins has a suppressive effect on the HIV-1 Vpr activities. In addition, there appears to be an antagonistic interaction between the host cellular heat shock responses and HIV-1 Vpr. Based on these data, we propose that HSP27, and possibly other HSF1-mediated HSPs, have evolved as a component of the intrinsic cellular response to HIV infection and specifically to Vpr. However, these stress responses are normally not sufficient to suppress the Vpr activities because Vpr actively counteracts activation of HSPs, likely via transcriptional inhibition. The finding that Vpr suppresses host innate antiviral responses involving HSPs suggests new antiviral therapeutic strategies aimed at blocking this effect of Vpr. This idea is certainly supported by our finding that expression of either human *HSP27* or fission yeast *Hsp16* under the control of exogenous promoters significantly reduced the Vpr activities. Because these activities have been linked to such clinical manifestation of AIDS as activation of viral replication (40), suppression of host immune responses (10) and depletion of $CD4^+$ T-lymphocytes (7,9), HSP-targeted strategies may help reduce Vpr-mediated detrimental effects in HIV-infected patients.

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