Human immunodeficiency virus 1 (HIV-1) encodes a gene product, Vpr, that facilitates the nuclear uptake of the viral pre-integration complex in non-dividing cells and causes infected cells to arrest in the G2 phase of the cell cycle. Vpr was also shown to cause mitochondrial dysfunction in human cells and budding yeasts, an effect that was proposed to lead to growth arrest and cell killing in budding yeasts and apoptosis in human cells. In this study, we used a genetic selection in Saccharomyces cerevisiae to identify hexameric peptides that suppress the growth arrest phenotype mediated by Vpr. Fifteen selected glutathione S-transferase (GST)-fused peptides were found to overcome to different extents Vpr-mediated growth arrest. Amino acid analysis of the inhibitory peptide sequences revealed the conservation of a di-tryptophan (diW) motif. DiW-containing GST-peptides interacted with Vpr in GST pull-down assays, and their level of interaction correlated with their ability to overcome Vpr-mediated growth arrest. Importantly, Vpr-binding GST-peptides were also found to alleviate Vpr-mediated apoptosis and G2 arrest in HIV-1-producing CD4+ T cell lines. Furthermore, they colocalized with Vpr and interfered with its nuclear translocation. Overall, this study defines a class of diW-containing peptides that inhibit HIV-1 Vpr biological activities most likely by interacting with Vpr and interfering with critical protein interactions.

Human immunodeficiency virus 1 (HIV-1) Vpr gene product is a small (14 kDa) phosphorylated nuclear protein that is highly conserved among HIV-1, HIV-2, and simian immunodeficiency virus (for review, see Refs. 1 and 2). Several functional studies of Vpr have been demonstrated in vitro underlining the importance of this protein for HIV replication and pathogenesis. Vpr is packaged into viral particles, suggesting that it may play a role in early events during HIV-1 infection (3, 4). Indeed, some experimental evidence indicates that Vpr increases HIV-1 replication in non-dividing cells such as macrophages, possibly by facilitating with other viral components nuclear import of the large viral pre-integration complex (5–7). Interestingly, Vpr localizes predominantly to the nucleus in a variety of cell types and was found to contain two non-canonical nuclear localization signals located in the N terminus and the C-terminal part of the protein (5, 7–11).

Another function of Vpr has been shown to promote cell differentiation and growth arrest at the G2/M phase of the cell cycle (12, 13). This property of Vpr was proposed to enhance viral replication because HIV-1 transcription is presumably more active during the G2 phase of the cell cycle (14, 15). Vpr-mediated cell cycle G2 arrest can be observed in cells from distantly related eukaryotes including human and fission yeast (Schizosaccharomyces pombe) and was shown to occur through inhibitory phosphorylation of Cdc2/Cdk1 (16–19). In all eukaryotic cells, entry into mitosis is regulated by the phosphorylation status of Cdc2/Cdk1, which is phosphorylated by Myt1 and Wee1 protein kinases during G2 and rapidly dephosphorylated by the Cdc25C phosphatase to trigger entry into mitosis. Both Wee1 and Cdc25C activities are themselves regulated at the level of their subcellular localization as well as by upstream kinase/phosphatase networks (20). Indeed, recent genetic studies with fission yeast suggest that Vpr induces cell cycle G2 arrest through a pathway involving protein phosphorylation (21, 22). These observations suggest that Vpr targets a well conserved cellular pathway controlling the G2 checkpoint during cell cycle. However, although Vpr has been shown to interact with various host proteins (23–26), the molecular mechanism underlying Vpr-induced cell cycle G2 arrest in HIV-1-infected cells remains undefined.

In addition to nuclear targeting and cell cycle G2 arrest activities, Vpr was also shown to differentially regulate the occurrence of apoptosis in human cells. In particular, it has been reported that during active HIV-1 replication, Vpr can induce cell killing by apoptosis by a mechanism that is independent from cell cycle G2 arrest (15, 27–30). Moreover, several studies showed that Vpr is capable of regulating either positively or negatively, apoptosis depending on the level of protein expression or the state of immune activation (31, 32). More recently, Vpr was shown to induce mitochondrial membrane permeability dysfunction in both human and budding yeast (Saccharomyces cerevisiae), an observation that led some to propose that this effect might be responsible for Vpr-mediated...
apothesis in human cells and growth arrest and cell killing in budding yeasts (33–35). Jacotot et al. (33, 34) showed that the addition of extracellular Vpr or Vpr C-terminal peptides to human cells or isolated mitochondria could permeabilize mitochondria, leading to a decreased membrane potential and the release of cytochrome c and apoptosis-inducing factor. The major target for Vpr in the mitochondrial membrane appears to be the permeability transition pore complex given that this polypeptide corresponding to Vpr C-terminal was reported to be specifically adenine nucleotide translocator, a major component of the permeability transition pore complex (34). Experiments in budding yeast further support that Vpr induces cell killing through mitochondrial membrane permeability by interaction with permeability transition pore complex. Externally added Vpr kills budding yeast, and the same region of Vpr is required to kill both yeast and mammalian cells (33, 34). Even though the mechanism involved in Vpr-induced apoptosis and cell killing is still not fully understood, all of these studies indeed stress the importance of Vpr during HIV-mediated pathogenesis.

In the present study, we used a genetic selection in S. cerevisiae budding yeasts to select a panel of 15 glutathione S-transferase (GST)-fused hexameric peptides that suppress the growth arrest phenotype of HIV-1 Vpr. Sequence analysis reveals that a common di-tryptophan amino acid motif is conserved in all inhibitory peptides, suggesting that this motif is critical for the ability of GST-peptides to interfere with HIV-1 Vpr activity in budding yeasts. Mechanistic analyses indeed reveal that the peptide-mediated growth arrest inhibition is not the result of a lack of synthesis nor degradation of Vpr but is instead mediated via an interaction between GST-peptide fusion proteins and Vpr. Interestingly, expression of Vpr-binding GST-fused peptides in human CD4+ Jurkat T cell lines alleviated Vpr-mediated apoptosis and cell cycle G1 arrest upon infection with VSV-G-pseudotyped HIV-1 virus. Furthermore, intracellular localization analysis revealed that the inhibitory GST-peptides co-localized with HIV-1 Vpr in mammalian cells and interfered with the protein nuclear translocation. This yeast genetic selection system represents a novel approach of identifying peptide inhibitors of HIV-1 Vpr biological activities and provides information about amino acid motifs that may be present in Vpr-interacting cellular factors or downstream effectors.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Antiserum, and Chemicals—**The HIV-1 Vpr yeast expression plasmid (p424Gal1-Vpr) was constructed by inserting a PCR-generated BamHI-BamHI fragment containing a Vpr sequence (11) into a high copy yeast expression plasmid, p424Gal1, which harbors a galactose-inducible Gal1 promoter and a tryptophan selection marker (36). A random hexameric peptide library fused to the C terminus of a GST inert carrier protein (>10^10 independent transformants) was generated (see below for a description) in a plasmid containing a phosphoglycerate kinase promoter and a URA3-selectable marker. The constitutive expression of GST-peptide fusion proteins is under the control of a phosphoglycerate kinase promoter (37). To generate a plasmid capable of expressing GST-peptides in mammalian cells, an EcoRI-BstXI fragment encoding GST-peptide was obtained from phosphoglycerate kinase-based yeast expression plasmids and cloned into the mammalian cell expression vector pHBeo (38), which contains a SL3–5 murine leukemia retrovirus long terminal repeat (>441–53; +1 = site of transcription initiation) or pCDNA1.1 (Invitrogen). pBeo contains the Epstein–Barr virus origin of origin and directs the expression of the Escherichia coli gene encoding hygromycin B phosphotransferase (Hygr) using the herpes simplex virus thymidine kinase promoter and polyadenylation site. The Vpr/green fluorescent protein (GFP) dual-expression plasmids, SVCMV-R–GFP and SVCMV-R–GFP, were constructed by inserting a BamHI-BglII fragment containing a CMV promoter-GFP-poly(A) cassette derived from the pQ2BEZ plasmid, (Quantum Biotechnologies, Inc.) into the BamHI site of SVCMV-R+ or SVCMV-R expression plasmids (11). These plasmids were used to analyze Vpr-mediated G2 arrest in transient expression assays. The HIV-1 envelope-defective pseudoviruses containing HXB2R–Env and HzB2R–Env as well as the vesicular stomatitis virus envelope G glycoprotein expression plasmid, SVCMV-VSV-G, used in this study were previously described (15).

The rabbit anti-Vpr polyclonal serum was raised against bacterially expressed recombinant Vpr as described previously (40). The goat antibody directed against GA1 was purchased from Amersham Biosciences. Fluorescein-conjugated mouse anti-goat IgG and the rabbit polyclonal mouse anti-rabbit antibodies were, respectively, purchased from Sigma Inc. and Jackson ImmunoResearch Laboratories Inc. Galactose, raffinose, glucose, and propidium iodide were purchased from Sigma. The annexin V–fluorescein isothiocyanate kit was purchased from Roche Molecular Biochemicals.

**Library Construction—**A peptide library was synthesized from an oligonucleotide containing 18 randomized nucleotides with SfiI and XhoI restriction sites at the 5′ and 3′ ends, respectively, 5′-AGTAGGCTTG-AGGCGGCTTNNKKNKKNNKKKGTCTAGAGTCGGC-3′ (41, 42). Randomized codons, designated NNK where N is either A, C, G, or T, and K is G or T produce a population of peptide sequences as described (41, 42). From the NNK motif, 32 possible codons can be generated that encode all amino acids and only one of the 3 possible stop codons. An oligonucleotide with complementarity to the 3′ end of the library oligonucleotide (5′-GCCGATCCTCTTAG) was annealed, and the complete complementary strand was synthesized using the Klenow enzyme in the presence of all four deoxynucleotide triphosphates. The resulting double-stranded product was restricted with SfiI and XhoI enzymes and inserted into identically restricted GST expression plasmid. The library was transformed into E. coli, and plasmid DNAs were harvested by alkaline lysis of bacterial colonies. The library is composed of greater than 1 × 10^10 independent transformants and at least 95% of the plasmids contained inserts encoding GST hexameric peptide fusions (41). The hexamers are joined to the C terminus of GST by a linker composed of Gly-Leu-Ser-Gly-Pro residues. The nucleotide sequence at the GST-hexamer fusion junction is AAA GCC CTG AGC GCC CTG (NNK), GTC TAG A. Thus the peptide sequence at the fusion junction is YGLSGP(X)_V-stop.

**Yeast Strains and Genetic Selection of Anti-Vpr GST-Peptides—**The S. cerevisiae yeast strain used in this study was the protease-deficient HP16 strain (MATα ura3–52 his3A1 leu2 trp1A36 prb1–1122 pep4–3 prc1–407) (43). Plasmid transformation was performed using the lithium acetate method (44). To test for Vpr expression and Vpr-mediated phenotypic changes, HP16 cells transformed with p424Gal1-Vpr were grown in selective medium (SC-trp (45) containing galactose (2%). To co-express Vpr and GST-hexameric peptides in yeast, the HP16 yeast strain harboring the p424Gal1-Vpr plasmid was re-transformed with the GST-peptide library, plated on solid medium with galactose, and selected for Ura and Trp independence. After 6–8 days at 30 °C, colonies were further analyzed. GST-peptide plasmid DNAs were rescued from positive clones as described previously (46). DNA-sequencing analysis of isolated plasmids encoding GST-peptide was performed with the denatured DNA-fragment analysis method (47). To test for Vpr expression and Vpr-mediated phenotypic changes, HP16 cells transformed with p424Gal1-Vpr were grown in selective medium (SC-trp (45) containing galactose (2%). To co-express Vpr and GST-hexameric peptides in yeast, the HP16 yeast strain harboring the p424Gal1-Vpr plasmid was re-transformed with the GST-peptide library, plated on solid medium with galactose, and selected for Ura and Trp independence. After 6–8 days at 30 °C, colonies were further analyzed. GST-peptide plasmid DNAs were rescued from positive clones as described previously (46). DNA-sequencing analysis of isolated plasmids encoding GST-peptide was performed with the denatured DNA-fragment analysis method (47).

**Cell Lines, Transfections—**Human CD4+ Jurkat T cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. Jurkat cell lines stably expressing GST-peptides (GST, GST-p4, -p12, and -p18) were established by electroporating 10 µg of the linearized pHBeo construct encoding the corresponding GST-peptide. Drug-resistant Jurkat cells were selected with growth medium containing hygromycin B at a concentration of 500 µg/mL. Primary human epithelial 293T cells and the African green monkey kidney COS-7 cell line were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. For transfection of 293T cells and COS-7 cells, the standard calcium phosphate coprecipitation technique was used, as previously described (11). All cells were maintained at 37 °C in 5% CO2.

**Virus Preparation and Viral Infection—**VSV-G pseudotyped HIV-1 virus preparations were generated by co-transfection of 293T cells with 10 µg of envelope-defective HIV-1 proviral DNAs and 15 µg of the VSV-G expression plasmid SVCMV-VSV-G using the calcium phosphate coprecipitation method (15). Forty-eight hours post-transfection, cell-free virus was collected by ultracentrifugation in a Beckman 60 Ti rotor for 1 h to pellet pseudotyped virus. Virus was resuspended in RPMI medium and filtered through a 0.45-µm-pore-size filter (Costar, Cambridge, MA). Virus stocks were titrated using the MAGI assay (47). To infect Jurkat cells, 0.25 × 10^6 cells were incubated
with VSV-G pseudotyped HIV-1 virus at different multiplicities of infection (m.o.i.) for 12 h. Infected cells were washed, cultured for another 36 h, and harvested for cell cycle analysis and detection of apoptosis.

**Cell Cycle Analysis and Annexin V/Propidium Iodide Double Staining**—To detect apoptosis in infected cells, the annexin V-fluorescein isothiocyanate assay was performed as recommended by the manufacturer (Roche Molecular Biochemicals, Inc.). Briefly, 0.25 × 10^6 infected cells were washed once with PBS and then resuspended in annexin V binding buffer (2.5 μg/ml annexin V-fluorescein isothiocyanate, 10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 1 μg/ml propidium iodide). After 10–15 min of incubation, stained cells were washed twice with binding buffer, resuspended in binding buffer containing 1% paraformaldehyde, and subsequently analyzed by FACScan. To perform cell cycle analysis, infected cells were washed once with PBS and resuspended in 80% ethanol for 30 min on ice. For the 293T cells co-transformed with Vpr/GST-fusion protein expression plasmid and the GST-peptide expression plasmids, GFP-positive cells were sorted out by fluorescence-activated cell sorter at 48 h post-transfection, washed once with PBS, and re-suspended in 80% ethanol for 30 min on ice. After an additional wash, cells were treated with 180 units/ml RNase A and subsequently stained with 30 μg/ml propidium iodide in 1 ml of PBS at 37 °C for 30 min. The DNA content was then analyzed by FACScan using the Consort 30 software. At least 10,000 events were collected for flow cytometry. Data acquisition and analysis were performed with the Cell Quest software (BD Biosciences). Samples were gated to exclude debris and clumps, and electronic compensation was used to remove residual spectral overlap. The mathematical model MODFIT was used to calculate the proportions of cells in the G2/M phases and G1 phase of the cell cycle. For simplicity, G2/M/G1 ratios have been provided.

**Immunoblot Analysis, Metabolic Labeling, and GST Pull-down Assay**—To examine expression of Vpr and/or GST-peptide fusions in yeast, HP16 co-transformants grown in suspension were pelleted by centrifugation at 15,000 rpm for 10 min and lysed in buffer (50 mM Tris-HCl, pH 7.4, 400 mM NaCl, 0.5% Nonidet P-40) using three cycles of vortexing. Cell debris was removed by centrifugation (15,000 rpm, 30 min), and cell lysates were incubated with 80 μg/ml of glutathione-activated Sepharose 4B beads (Amersham BioSciences) for 2 h at 4 °C. The membrane was incubated with goat polyclonal antibodies against GST or rabbit polyclonal anti-Vpr antibodies overnight at 4 °C and then probed at room temperature with horseradish peroxidase-linked sheep anti-goat or anti-rabbit antibodies (Amersham Biosciences) for 2 h. The membrane was washed extensively and revealed using a sensitive enhanced chemiluminescence detection system (ECL detection kit, Amersham Biosciences).

To detect the interaction of Vpr and GST-peptide in yeast, HP16 yeast cell extracts expressing GST-peptide and Vpr were radiolabeled with 150 μCi of 35S-Translabel (ICN Inc.) for 6 h at 30 °C. After labeling, cell extracts (5 μl of CHAPS buffer (250 mM NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% CHAPS (Sigma)) supplemented with a protease inhibitor mixture (Roche Molecular Biochemicals) by vortexing with 0.6 g of glass beads. After a centrifugation at 3,000 rpm in a Sorvall SS 3000 rotor for 10 min at 4 °C, supernatants were collected and used for GST pull-down assay (7). Briefly, 200 μl of yeast lysate was mixed with 700 μl of column buffer (20 mM Tris-Cl, pH 7.4, 200 mM NaCl, 1 mM EDTA supplemented with a protease inhibitor mixture (Roche Molecular Biochemicals)) and incubated with 80 μl of glutathione-Sepharose 4B beads (Amersham Biosciences) for 2 h at 4 °C. The glutathione-Sepharose 4B beads were sedimented by centrifugation and washed 3 times in 500 μl of column buffer, and the radiolabeled protein complexes were eluted with 100 μl of glutathione buffer (100 mM reduced glutathione (Roche Molecular Biochemicals), 120 mM NaCl, 100 mM Tris-HCl pH 8.5) by shaking at 4 °C for 1 h. Eluted protein complexes were loaded onto 12.5% SDS-PAGE, and the presence of GST-peptides and Vpr was revealed by autoradiography. Meanwhile, to detect the total amounts of Vpr, 200 μl of yeast lysate was immunoprecipitated with rabbit anti-Vpr antibodies as described (11). Results as described in the manuscript are shown in Supplementary Data S1 and Supplementary Data S2.

**RESULTS**

**Selection of GST-fused Hexameric Peptides That Suppress Vpr-mediated Growth Arrest in S. cerevisiae**—Several studies show that HIV-1 Vpr causes cell growth arrest and cell killing in budding yeast by a mechanism that appears to involve mitochondrial dysfunction (33, 35, 48). S. cerevisiae was chosen as a host to carry out the genetic selection of peptides interfering with Vpr biological activity for several reasons, which include the following: 1) Vpr induces a well-defined phenotype in budding yeast that can be studied using defined genetic backgrounds; 2) peptide interfering with Vpr biological activity in this system may provide valuable information on the molecular mechanism underlying Vpr-mediated mitochondrial dysfunction; 3) the peptide library was initially designed and constructed for expression in budding yeast given the availability of proven expression, induction, and selection systems. We generated a yeast expression plasmid (p424Gal1-Vpr) encoding HIV-1 Vpr under the control of the galactose-inducible GAL1 promoter and transformed HP16 S. cerevisiae strain to confirm that Vpr induced growth arrest in budding yeast under our experimental conditions. In parallel, the empty p424Gal1 vector was transformed as a negative control. After growing for 2 days in Vpr non-inducible selective medium (Trp⁻, 2% raffinose (raf⁻)), yeast cells transformed with either the p424Gal1-Vpr or p424Gal1 plasmid showed comparable growth rates (Fig. 1A, left panel). However, when grown in the Vpr-inducible medium (Trp⁻, 2% galactose (gal⁺)), yeast cells transformed with p424Gal1-Vpr exhibited a significant growth defect when compared with cells transformed with the p424Gal1 control plasmid (Fig. 1A, right panel). To test whether this effect correlated with Vpr induction, Vpr expression in both p424Gal1-Vpr- and p424Gal1-transformed yeasts was evaluated by Western blotting using anti-Vpr antibodies. Results of Fig. 1B reveal that Vpr expression is only detected in the sample derived from p424Gal1-Vpr-transformed yeast cells grown in Vpr-inducible medium (lane 4). These results confirm that expression of HIV-1 Vpr in S. cerevisiae HP16 cells induces a strong growth arrest phenotype.

We next used this yeast system to screen a GST-fused hexameric peptide (GST-peptide) library to genetically select peptides inhibiting Vpr-induced growth arrest. The construction and the organization of the GST-fused hexameric peptide library are described under “Experimental Procedures.” The peptide library (a library of 1 × 10⁶ GST-peptides) was trans-
Hexameric Peptides Inhibiting HIV-1 Vpr

Genetic selection of GST-peptide inhibiting Vpr-mediated yeast growth arrest. A, HP16 strain of S. cerevisiae yeast was transformed with plasmids p424Gal1-Vpr or p424Gal1 and grown in selective/Vpr non-inducible (trp*, ura*) or inducible (trp*, gal*) medium for 2 days. Yeast growth was then monitored by measuring each yeast cell culture density by spectrophotometric analysis at a wavelength of 600 (A600). B, yeast cultures grown in either Vpr non-inducible or inducible selective media were collected and lysed in Nonidet P-40 lysis buffer (in the presence of glass beads). Expression of Vpr in cell lysates was detected by Western blotting with anti-Vpr antibodies. C, HIV-1 Vpr-expressing HP16 yeasts were transformed with a GST-fused hexameric peptide library and selected in agar plates in Vpr-inducible conditions (trp*, ura*, gal*). After two rounds of selection on agar plates, the growing clones were cultured in doubly selected/Vpr-inducible liquid medium (trp*, ura*, gal*), and the growth of each yeast clone was evaluated after 3 days of incubation by measuring the cell density by spectrophotometry at a wavelength of 600 (A600). The asterisk indicates that clone 10 (C10) was found to express two distinct GST-peptides. These data are representative of results obtained in two independent experiments. D, expression of GST-peptides and Vpr in yeast. HP16 yeasts co-transformed with Vpr and each GST-peptide were first cultured in Vpr-non-inducible selective medium for 3 days and then grown in Vpr-inducible selective medium overnight. Yeasts were then collected and lysed, and similar amounts of protein (500 μg) were analyzed by SDS-PAGE and Western blotting using specific anti-GST upper panel or anti-Vpr antibodies lower panel. M, non-transformed yeast. The GST-p9 and p10 were, respectively, expressed from two distinct GST-peptide phosphoglycerate kinase plasmids that contained DNA fragments encoding each of the two GST-peptides found in clone10 (C10) (as indicated in panel C).

Formed into HP16 yeast cells harboring the p424Gal1-Vpr expression plasmid. Transformants containing both Vpr and GST-peptide expression plasmids were selected for tryptophan and uracil independence in medium supplemented with galactose (2%). After 6–8 days of culture, approximately 50 growing colonies were randomly selected from ~10 × 10⁶ transformants. After multiple steps of re-selection, 14 yeast clones, designated C1, C2, C3, C4, C5, C6, C7, C10*, C11, C12, C13, C16, C17, and C18, were shown to proliferate effectively in Vpr-inducible liquid medium (trp*, ura*, gal*), whereas yeasts expressing both Vpr and GST alone exhibited a strong growth defect (Fig. 1C and data not shown for C17).

False positive results are common to genetic selections and screens that rely on transcription of one component of the genetic system. A likely false positive result in our system is a GST-peptide that interferes with Vpr expression from the p424Gal1-Vpr plasmid. To test this possibility, the levels of Vpr and GST-peptides were evaluated in each selected clone by Western blot. Results with anti-GST antibody reveal that similar amounts of GST or GST-peptides were expressed in co-transformed yeast cells (Fig. 1D, upper panel). In parallel, immunoblotting with anti-Vpr antibodies clearly shows that abundant and comparable amounts of Vpr are also detected in each co-transformant but not in non-transformed yeast (Fig. 1D, lower panel). These results demonstrate that expression of the selected GST-peptides suppressed to different extent Vpr-mediated growth arrest by a mechanism that did not involve a negative modulation of Vpr levels in the selected yeast clones.

Sequence Analysis Reveals That a Common Double-tryptophan Motif Is Conserved in All Selected GST-fused Peptides—Plasmid DNAs encoding GST-peptides were rescued, and their nucleotide sequence at the junction between GST and the hexameric peptide library was determined. We found that the plasmid isolated from yeast clone C10 encoded two GST-fused peptides, each of them driven by their own phosphoglycerate kinase promoter. After separation and subcloning into the phosphoglycerate kinase-GST plasmid, the clones were re-designated GST-p9 and GST-p10, respectively (as shown in Fig. 1D, lower panel, and 2A). Sequence analysis reveals that all 15 selected GST-peptides (GST-p) contain a conserved di-tryptophan (diW) motif, suggesting that the presence of this motif within peptides may be critical for their ability to suppress Vpr-mediated growth arrest activity (Fig. 2A). Interestingly, the amino acid sequence of four GST-peptides, GST-p10, GST-
Panel A shows a schematic structure of a GST-fused peptide with the deduced amino acid sequence of the peptide moieties below. The peptide library was linked to the C terminus of the GST protein through a linker comprising six amino acids (KGLSGP), whereas the peptide C terminus was linked to a valine residue. B, schematic representation of the computer-predicted amphipathic structure of peptide 4, 16, and 18. Hydrophobicity plots were determined according to Kyte and Doolittle using the MacVector software (International Biotechnologies, New Haven, CT).

Another observation was that most peptides were rich in hydrophobic amino acids, especially at their C terminus (Fig. 2A), suggesting that in addition to the diW motif the hydrophobicity of the peptides may also be required for their inhibitory activity. Interestingly, even though GST-p18 and GST-p4 contain a WXWW sequence, GST-p18 exhibits a stronger inhibitory activity toward Vpr-mediated growth arrest than GST-p4 (Fig. 1C and Fig. 3A). Computer analysis predicts that GST-p4 exhibits a high hydrophilicity at the C terminus as compared with both GST-p18 and GST-p16, which contain a hydrophobic C-terminal sequence (Fig. 2B). We conclude from these results that the presence of a diW motif and the preservation of a hydrophobic C terminus may be important parameters governing the selected peptide anti-Vpr activity. However, we cannot exclude the possibility that extension of the C terminus relative to the diW motif position, such as in GST-p4, may also affect the peptide inhibitory activity.

Selected GST-Peptides Specifically Inhibit the Effect of Vpr on Cell Growth and Morphology—To test whether the selected GST-peptides specifically affect HIV-1 Vpr activity or alternatively have a general effect on cell proliferation, we selected a panel of 8 representative diW-containing GST-peptides including GST-p1, -p4, -p9, -p10, -p12, -p16, -p17, -p18 and re-transformed purified plasmid DNAs encoding these 8 anti-Vpr GST-peptides into the HP16 yeast strain harboring p424Gal1-Vpr. Transformant suspensions of similar cell densities were then serially diluted (10) and spotted onto either a Vpr non-inducible plate (raffinose) or a Vpr-inducible plate (galactose), and their growth was evaluated after an incubation of 3–5 days (Fig. 3A). In the absence of Vpr expression, yeast cells constitutively expressing each GST-peptide or the GST control grew at similar rate (Fig. 3A, a), thus indicating that expression of these GST-peptides per se had no general effect on yeast proliferation. As expected, when HIV-1 Vpr expression was induced, yeast co-expressing Vpr and the GST control exhibited a profound growth arrest, whereas yeast expressing the GST control only, grew efficiently (Fig. 3A, b, compare lanes 2 and 3 to lane 1). In contrast, co-expression of the selected GST-peptides was shown to overcome the yeast growth arrest mediated by Vpr albeit to different extent (Fig. 3A, b, compare lanes 4–11 to lanes 2 and 3). GST-p18, -p17, -p16, and -p12 exhibited the strongest inhibition, whereas interestingly, GST-p4 displayed the weakest inhibitory activity. These results strongly indicate that the selected GST-peptides specifically inhibit the activity of Vpr that mediates cell growth arrest in budding yeasts.

In addition to mediating cell growth arrest, Vpr has also been shown to induce structural or morphological changes in S. cerevisiae (48, 50). To determine whether the most potent inhibitory GST-peptides (GST-p18 or GST-p16) could interfere with this Vpr effect, HP16 yeast cells either expressing the GST control or co-expressing Vpr and GST or GST-p16 or -p18 were cultured in Vpr-inducible medium for 3 days and examined by light microscopy. Results show that expression of the GST control did not affect cell morphology (Fig. 3B, a) as compared with non-transformed cells (data not shown). In contrast, profound morphological changes were observed when Vpr was co-expressed with GST. These Vpr-induced morphological changes were highly polymorphic, including enlarged, spherical, and shrunken cells (Fig. 3B, b). When Vpr was co-expressed with GST-p16 or GST-p18, most cells exhibited a normal size and morphology, and very few shrunken cells were present in the cultures (Fig. 3B, c and d). These results indicate
that in addition to suppressing Vpr-mediated growth arrest, GST-p18 and GST-p16 can also strongly attenuate Vpr-mediated morphological changes in budding yeast.

Interaction of Di-W-containing GST-Peptides with Vpr—In an attempt to elucidate the mechanism(s) underlying the GST-peptide inhibitory effect, we investigated the ability of a panel of representative diW-containing GST-peptides, including GST-p4, GST-p12, GST-p16, and GST-p18, to interact with Vpr in yeast cells by GST pull-down. These GST-peptides were primarily selected on the basis of their inhibitory effect on Vpr-mediated growth arrest. HP16 yeast cells co-expressing HIV-1 Vpr and GST or GST-peptide (as indicated) were radio-labeled with 35S-Translabel for 6 h, and lysed with CHAPS lysis buffer. GST or GST-peptides complexes were then pulled down with glutathione-Sepharose 4B. After extensive washes, protein complexes were eluted with 100 mM glutathione and the radiolabeled GST, GST-peptide complexes were separated by SDS-PAGE and revealed by autoradiography (panel A). In parallel, total amounts of Vpr in each sample were immunoprecipitated with anti-Vpr antibodies and analyzed by SDS-PAGE and autoradiography (panel B). As controls, yeast cells expressing or not Vpr (panels A and B, lanes 6 and 7) were also labeled and analyzed using the same procedure. The positions of GST, GST-peptide, and Vpr are indicated on the right side of the autoradiogram.

![Fig. 3](image-url)

**Fig. 3.** Selected GST-peptides inhibit specifically the effect of Vpr on cell growth and morphology in budding yeast. A, a panel of plasmids encoding GST-peptide or the GST control (as indicated on the left side of the panel) were transformed into S. cerevisiae HP16 strain that contained either p424Gal1-Vpr or p424Gal1 (as indicated). Yeast co-transformants were selected and grown in non-inducible selective medium (trp⁺, ura⁻, raf⁻) for 2 days. Similar amounts of yeast (0.5 A₆₀₀) were then serially (10⁻²) diluted, spotted onto either Vpr non-inducible selective agar plates (trp⁺, ura⁻, raf⁻) (left panel) or Vpr-inducible selective agar plates (trp⁺, ura⁻, gal⁻) (right panel) and incubated for 3–5 days to evaluate their growth rates. These data are representative of results obtained in two independent experiments. B, the S. cerevisiae HP16 strain expressing either GST alone (a) or both Vpr and GST (b), GST- p16 (c), or GST-p18 (d) were cultured in Vpr-inducible selective medium for 3 days and examined by light phase microscopy.

![Fig. 4](image-url)

**Fig. 4.** Selected GST-peptides interact directly with HIV-1 Vpr. HP16 yeast cells co-expressing HIV-1 Vpr and GST or GST-peptide (as indicated) were radio-labeled with 35S-Translabel for 6 h and lysed with CHAPS lysis buffer. GST or GST-peptides complexes were then pulled down with glutathione-Sepharose 4B. After extensive washes, protein complexes were eluted with 100 mM glutathione and the radiolabeled GST, GST-peptide complexes were separated by SDS-PAGE and revealed by autoradiography (panel A). As controls, yeast cells expressing or not Vpr (panels A and B, lanes 6 and 7) were also labeled and analyzed using the same procedure. The positions of GST, GST-peptide, and Vpr are indicated on the right side of the autoradiogram.

5), detectable amounts of Vpr were pulled down by GST-p18, GST-p16 GST-p12, and GST-p4 (Fig. 4A, lanes 1–4). Furthermore, no protein corresponding to Vpr was pulled down by these GST-peptides when yeast cells were grown in Vpr non-inducing conditions, thus confirming that the co-purified 14kDa bands are indeed Vpr (data not shown). Interestingly, GST-p18 was found to pull down the largest amount of Vpr (quantitative analysis of the protein bands by scanning densitometry indicates that the ratio of bound Vpr over total Vpr is ~2–3-fold higher for GST-p18 as compared with GST-p12 and -p16, respectively), whereas the amounts of Vpr associated with GST-p4 were the lowest (Fig. 4A, compare lane 1 to lanes 2–4). As expected, no radioactive bands corresponding to Vpr or GST-peptides were detected in lysates prepared from yeast harboring p424Gal1-Vpr only and grown under Vpr-inducing or non-inducing conditions (Fig. 4A, lanes 6–7). To rule out the possibility that the different amounts of Vpr bound to GST-peptide are due to variable levels of Vpr expression, the same samples were immunoprecipitated with anti-Vpr antibodies, and the immunocomplexes were analyzed by SDS-PAGE and autoradiography. Results show that comparable amounts of Vpr were expressed in each yeast transformant grown in Vpr-inducing conditions (Fig. 4B, lanes 1–5 and 7). Overall, these
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results clearly indicate that diW-containing GST-peptides p18, p16, p12, and p4 interact with Vpr, albeit with different efficiency. GST-p18 showed the highest Vpr binding efficiency and displayed the stronger inhibitory effect on Vpr-mediated growth arrest. This finding strongly suggests that diW-containing GST-peptides suppress Vpr-mediated growth arrest by interacting with Vpr.

Expression of Vpr-binding GST-Peptides in the Human CD4+ Jurkat T Cell Line Alleviates Vpr-mediated Apoptosis and Cell Cycle G2 Arrest upon Infection with VSV-G-pseudotyped HIV-1 Virus.—To investigate whether Vpr-binding GST-peptides could impair Vpr-mediated apoptosis and cell cycle G2 arrest in human CD4+ T cells, we generated CD4+ Jurkat T cell populations expressing GST-peptides including GST-p4, -p12, and -p18 as well as the GST control. Each cell population was analyzed for its ability to express the corresponding transgene by semiquantitative reverse transcription-PCR since detection of GST-peptide fusion proteins by Western blotting using anti-GST antibodies did not lead to clear results. This is likely due to the fact that GST-peptide fusion protein expression in these Jurkat cell populations was at the limit of immunoblot detection levels. Results of Fig. 5A reveal that GST-p4, -p12, and -p18 and GST mRNAs were detected in the corresponding Jurkat T cell populations, indicating that the transgenes were adequately transcribed. Expression of the GST-peptides did not appear to affect Jurkat cell growth or morphology as compared with the GST-expressing Jurkat cell population control (data not shown).

To test the effect of each GST-peptide on Vpr-mediated apoptosis and cell cycle G2 arrest, each GST-peptide expressing Jurkat T cell population was infected with Vpr+ (HxBrur+/E−) or Vpr− (HxBrur-/E+) VSV-G pseudotyped HIV-1 viruses at m.o.i. of 0.125 and 0.25. Infected cells were collected and analyzed for cell cycle and apoptosis. The use of VSV-G-pseudotyped virus allowed us 1) to efficiently infect each cell population and 2) to examine the effect of GST-peptides on Vpr-mediated apoptosis and cell cycle G2 arrest in the absence of viral spread and without the complication of envelope-mediated cell death. Results from Fig. 5B clearly show that expression of GST-peptides had no significant effect on the percentage of apoptotic cells resulting from infection with Vpr-defective pseudotyped virus at both m.o.i. As previously reported, expression of Vpr was found to enhance apoptosis as revealed by the increased number of annexin V-positive apoptotic cells in Vpr−-pseudotyped virus-infected GST-expressing Jurkat cell control cultures at both 0.125 and 0.25 m.o.i. In contrast, Jurkat cell populations expressing GST-p12 or p18 exhibited a significant reduction of annexin V-positive cells upon infection with Vpr−-pseudotyped virus as compared with the GST control especially at the lower m.o.i. (0.125) (Fig. 5B). GST-p4 did not show any significant inhibition of Vpr-mediated apoptosis. In parallel, Vpr-mediated cell cycle G2 arrest was also evaluated. Results of Fig. 5C clearly show that, whereas GST-p12 or p18 did not affect the cell cycle profile of Vpr−-pseudotyped HIV-1-infected Jurkat cell populations, expression of these GST-peptides attenuated Vpr-mediated cell cycle G2 arrest during Vpr− HIV-1 infection as compared with the GST control (Fig 5C). Unexpectedly, Jurkat cells expressing GST-p4 were also found to be less susceptible to Vpr-mediated cell cycle G2 arrest as compared with the GST-expressing Jurkat cell control, suggesting that GST-p4 retained some ability to inhibit Vpr activity on the cell cycle. Overall, these results indicate that expression Vpr-binding GST-peptides in human Jurkat T cells alleviates the apoptosis and the cell cycle G2 arrest mediated by HIV-1 Vpr. Similar results were obtained when GST-peptides where expressed in HeLa cells and infected with VSV-G pseudotyped HIV-1 virus (data not shown) or when 293T cells transiently overexpressing Vpr and GST-peptides where analyzed for Vpr-mediated cell cycle G2 arrest (Fig. 6).

Intracellular Localization of HIV-1 Vpr- and diW-containing GST-Peptide Fusions.—Previous studies have shown that HIV-1 Vpr localizes in the nucleus of mammalian cells when expressed in the absence of other viral proteins (11, 51, 52). To investigate the effect of Vpr-binding GST-peptides on Vpr nuclear localization, we individually expressed GST, GST-p4, -p16, or GST-p18 or co-expressed them with Vpr in COS-7. COS-7 cells have been previously shown to be responsive to Vpr-mediated cell cycle G2 arrest and apoptosis (53). They also have the interesting feature of having a well delineated nucleus and
cytoplasm, which facilitates intracellular localization analysis using immunofluorescence. Cells were fixed, labeled with anti-GST and/or anti-Vpr antibodies, and analyzed by indirect immunofluorescence and laser confocal microscopy 48 h post-transfection. When GST was expressed alone in COS-7 cells, the protein exhibited a diffuse staining pattern and localized both in the cytoplasm and the nucleus. (Fig. 7A, a). In contrast, the GST-peptide fusions, GST-p18 and GST-p16, were clearly found to be excluded from the nucleus and were shown to primarily accumulate in a perinuclear region within the cytoplasm (Fig. 7A, b and c), suggesting that the presence of diW motif-containing peptides at the C terminus of GST prevents GST diffusion into the nucleus. When GST was co-expressed with Vpr, co-staining results and confocal laser microscopy analysis showed that GST was still distributed both in the nucleus and the cytoplasm (Fig. 7B, a), whereas Vpr was predominantly located in the nucleus (Fig. 7B, b). Interestingly, even though both proteins were located in the nucleus, there was no apparent co-localization (Fig. 7B, c). In contrast, when GST-p16 or GST-p18 were co-expressed with Vpr, a clear cytoplasmic co-localization was observed (Fig. 7B, f and i). In the presence of GST-p18 or p16, Vpr nuclear localization pattern drastically changed (Fig. 7B, compare b with c and h). Intensive Vpr staining was observed at the periphery of the nucleus rather than in the nucleoplasm in the presence of GST-p18 or GST-p16 suggests that these peptides, by interacting with Vpr, may interfere with its nuclear translocation.

DISCUSSION

In this study, we have taken advantage of the growth arrest phenotype induced by Vpr in budding yeast to screen a GST-fused hexameric peptide library for GST-peptide fusions capable of inhibiting Vpr cell growth arrest activity. Fifteen GST-peptides that had the capacity to overcome Vpr-mediated growth arrest were identified using this genetic selection system. Given that the G$_t$ to M transition of budding yeast is regulated differently than in fission yeast or mammalian cells (inhibitory phosphorylation of the cyclin-dependent kinase Cdc28 of budding yeast does not play a major role in this transition (48, 54, 55)), it is unlikely that the selected peptides overcome Vpr-mediated budding yeast growth arrest by interfering with specific interactions between Vpr and cellular components regulating inhibitory phosphorylation of the cyclin-dependent kinase and the G$_t$-M transition, as was demonstrated in mammalian cells and fission yeast (16, 17, 19). Rather, intracellular expression of Vpr in budding yeast was shown to induce growth defects by a mechanism that appears to involve mitochondrial dysfunction (35). Macreadie et al. (35) find that constitutive production of Vpr in S. cerevisiae caused a respiratory deficiency due to effects on several mitochondrial enzymes. This deficiency caused some interference with yeast growth on fermentable carbon sources such as glucose but caused a complete block to growth on non-fermentable carbon sources such as glycerol or ethanol, where mitochondrial respiratory function is required. In addition, when added exter-

![Image](image.png)
nally, Vpr was found to cause yeast cell killing by a mechanism that involves defects in mitochondrial membrane permeability (33, 34). Although in both cases experimental evidence suggests that Vpr may act through the mitochondria, it still unclear whether intracellular yeast growth arrest and external cytotoxic effects are related (33, 35, 56). Nevertheless, these data strongly suggest that the GST-peptides identified in this study were selected on the basis of their ability to prevent or overcome Vpr-mediated mitochondrial dysfunction.

Using the GST pull-down binding assay, we have clearly showed that a set of representative GST-peptides interacted with Vpr, however, with different efficiencies. Interestingly, the growth arrest inhibitory effect mediated by the GST-peptides correlated very well with their Vpr binding efficiency. GST-p18 was found to have the strongest inhibitory effect (Fig. 3A) and was shown to strongly bind Vpr (Fig. 4A). In contrast, GST-p4 was found to have the weakest effect on Vpr-mediated growth arrest (Fig. 3A) and the lowest Vpr binding efficiency (Fig. 4A). These results strongly suggest that the interaction of the GST-peptides with Vpr interferes with its ability to induce a growth arrest in budding yeasts.

Sequence analysis of the GST-peptides exhibiting anti-Vpr activity revealed that they all contained a conserved diW motif. The conservation of such a motif within all selected peptides strongly suggest that it may be critical for the peptide ability to interact with Vpr and inhibit its biological activity. Indeed, randomly selected GST hexameric peptides that did not contain a diW motif were not found to inhibit Vpr-mediated growth arrest in budding yeast (data not shown). In addition, the most potent GST-peptides displayed a stretch of hydrophobic residues at their C terminus that might also confer selected peptides with some inhibitory activity (Fig. 2). However, the exact contribution of the diW motif and the hydrophobic C terminus to the peptide anti-Vpr activity remains to be determined. Interestingly, among 15 selected GST-peptides, four (GST-p16, -p17, -p13, and -p10) harbored a previously reported WXXF motif (49). The WXXF motif was shown to be a Vpr-interacting domain and was found to be present in the Vpr-interacting protein uracil DNA glycosylase (49). Several studies have also shown that fusion of WXXF motif to heterologous protein al-
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Because different functions of Vpr may optimize HIV-1 replication and contribute to HIV-1 pathogenesis in vitro, this protein has been proposed to be a target for the development of antiviral strategies. Several studies report different strategies to inhibit Vpr functions during HIV-1 replication, such as Vpr dominant mutant (R73S) (62), antagonist of the glucocorticoid receptor (RU486) (63), and pentoxifylline (64). In this study, we took advantage of a genetic selection system in budding yeast that allows the in vivo identification of hexameric peptide inhibitors that have functional relevance since they inhibit the function of target proteins, in this case Vpr, intracellularly. Overall, this study demonstrates that this genetic selection system provides a powerful tool for the rapid identification of potent inhibitors of biological processes in large combinatorial libraries.

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