Interplay between HIV-1 Vpr and Sp1 Modulates p21\textsuperscript{WAF1} Gene Expression in Human Astrocytes*

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The Vpr (viral protein R) of human immunodeficiency virus, type 1, which is expressed during the late stage of the viral infection, has received special attention because of its ability to control transcription of the human immunodeficiency virus, type 1, long terminal repeat and to influence cell cycle progression. Here we demonstrate that Vpr has the ability to regulate transcription of the cyclin-dependent kinase inhibitor, p21\textsuperscript{WAF1} (p21), one of the key regulators of the cell cycle, in human astrocytic cells. The results from transcription assays demonstrated that Vpr augments promoter activity of p21 through the GC-rich region located between nucleotides −84 and −74 with respect to the +1 transcription start site. Activation of p21 by Vpr required cooperativity of Sp1, which binds to the DNA sequence spanning −84 to −74. Results from bandshift assay revealed an increased level of Sp1 DNA binding activity in the presence of Vpr. Furthermore, Vpr was able to associate with Sp1 via the zinc finger domain located in the C-terminal region of Sp1. Functional studies revealed that the cooperativity between Vpr and Sp1 requires the zinc finger domain at the C terminus and the glutamine-rich domain at the N terminus of Sp1. Expression of p53 further enhanced the level of Vpr-Sp1-mediated transcription activation of p21 through the sequence spanning −84 to −74 and increased the DNA binding activity of Sp1 in the presence of Vpr. Results from glutathione S-transferase pull-down assay showed the association of Vpr with p53 in extracts containing Sp1. Altogether, the outcome of our functional and binding studies suggested that the physical interaction of Vpr with Sp1 and p53 could modulate transcriptional activity of p21.

The cyclin-dependent kinase inhibitor, p21\textsuperscript{WAF1} (p21), arrests cell cycle by modulating the activity of cyclin-dependent kinases and regulates DNA methylation by interacting directly with proliferating cell nuclear antigen, a subunit of DNA polymerase, and prevents DNA synthesis (1–4). p21 also plays important roles in the control of cell senescence, apoptosis, and differentiation (5–7). Expression of p21 is regulated by a wide range of proteins such as tumor suppressors including p53 and pRb (8, 9), growth factors, and several signaling proteins associated with cytokines including platelet-derived growth factor (4), tumor necrosis factor-α (11), interferon (12), progesterone (13), and transforming growth factor β (6). The association of these factors or their downstream responsive regulators with the specific DNA sequence spanning the p21 genome is important for their regulatory function upon p21. For example, p53, retinoid acid, vitamin D, and interferon may utilize the distal-specific cis-acting DNA motifs that extend between positions −2280 and −120 to stimulate transcription of the p21 gene (14). The other regulators such as transforming growth factor β, progesterone, phorbol esters, and phosphatase inhibitors mediate their effects on p21 gene expression via the proximal region of the promoter, which spans −120 to +1 (15). The proximal promoter contains consensus GC-rich motifs that may serve as binding sites for members of the Sp1 family of ubiquitous transcription factors (16).

Sp1 belongs to a zinc finger family of transcription factors and was first identified based on its ability to interact with the GC-rich motif of SV40 regulatory sequences (17–19). This protein plays a critical role in many cellular events by regulating expression of several other genes, including early embryonic development and the maintenance of terminal cell differentiation (20). The structure of Sp1 is composed of four domains, A–D, and a zinc finger region, which is involved in DNA-protein interaction (21). Domains A and B are the two major glutamine-rich regions essential for transcriptional activation. Although the role of domain C is not fully understood, it has been shown that the region spanning domain C may be involved in synergistic activation with steroid regulatory element-binding proteins (22). Domain D, by interacting with other activation domains, creates a configuration that maximizes their activity (23). The DNA binding and transcriptional activities of Sp1 are regulated by the state of phosphorylation that follows changes in the cell cycle (24). Sp1 protein is stabilized by O-linked glycosylation, which confers resistance to proteasome-dependent degradation (19, 25).

Sp1 has been shown to associate directly with members of the basal transcription complex such as TFIID and also physically interacts and functionally cooperates with several cellular transcriptional activators including YY1, GATA, NF-κB, Puro, E2F1, pRb, and SREBP-1 (26–32). Although initially considered a ubiquitous transcription factor that closely associates with the core promoter elements, recent studies (33, 34) have shown that Sp1 may conditionally regulate gene transcription by cooperating with various signaling pathways.

In addition to cellular factors, the partnership of Sp1 with several viral regulatory proteins (35) including the HIV-1

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The abbreviations used are: HIV-1, human immunodeficiency virus type 1; Vpr, viral protein R; Vpr-LR, Vpr-leucine-rich; Sp1, specificity protein 1; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; wt, wild type; ZF, zinc finger; LTR, long terminal repeat; CMV, cytomegalovirus; IP, immunoprecipitation.
accesory protein, Vpr, may have a functional consequence on viral and cellular gene expression (36, 37). Vpr is produced at the late phase of HIV-1 infection and plays a role in facilitating infection of nondividing cells such as macrophages (38). Furthermore, Vpr can inhibit cell cycle progression at the G2 phase, at least in part via inactivation of the cyclin-dependent kinase cdc2 and hyperphosphorylation and the concomitant suppression of cdc2-cyclin B kinase activity (38). Recent studies (39) have shown that by depolarizing the mitochondrial membrane, Vpr may induce apoptosis in host cells. In this study we have focused on the transcriptional activity of Vpr, and we assessed the ability of Vpr to cooperate with Sp1 in regulating transcription of p21 through the GC-rich motif identified between −84 and −74. We demonstrate that the interaction of Vpr and Sp1 can potentiate p53 to enhance transcription of p21 via the GC-rich motif.

MATERIALS AND METHODS

Plasmids—The p21-luciferase reporter plasmid was a gift from J. Manfredi (Mt. Sinai School of Medicine, New York), and its deletion mutons were generated in Datto et al. (15). The p21-CAT reporter plasmid and its deletion mutants were obtained from B. Vogelstein (The Johns Hopkins University, Baltimore) (40). CMV-Vpr full length as well as the Vpr protein lacking the leucine-rich domain (Vpr-LR) was described previously (36). The 6xSp1 reporter construct (CAT), containing cDNA driven by the minimal HIV-1LTR promoter, was kindly provided by K. T. Jeang (National Institutes of Health). The pPAC-Sp1 and CMV-p53 plasmids were described in Sawaya et al. (36). The pGEX-2T-Vpr and CMV-Tat were previously described in Sawaya et al. (41). The CMV Sp1 expression plasmid was kindly provided by R. Tijan (University of California, Berkley, CA). CMV-Sp1 deletion mutants were synthesized by PCR and cloned into EcoRiXhoI of pCDNA3. The sequences of the oligonucleotides used in PCR are as follows: a, 167/785, 5′-CTACCTGATTTCCACCAAAATATGCTGCTAG-3′; b, 266/785, 5′-CTCAGTATTTGCCACCAAAATATGCTGCTAG-3′; c, 653/785, 5′-CTCAGTATTTGCCACCAAAATATGCTGCTAG-3′; d, 785/167, 5′-AGCCATGATTTCCACCAAAATATGCTGCTAG-3′; e, 785/785, 5′-CTCAGTATTTGCCACCAAAATATGCTGCTAG-3′; f, 785/167, 5′-CTCAGTATTTGCCACCAAAATATGCTGCTAG-3′; g, 785/255, 5′-AGCCATGATTTCCACCAAAATATGCTGCTAG-3′; h, 350/167, 5′-CTCAGTATTTGCCACCAAAATATGCTGCTAG-3′.

RESULTS

We demonstrated previously the ability of Vpr to induce p21 at transcriptional and translational levels and that Vpr activation of the HIV-1 LTR is p21-dependent. We have also demonstrated that Vpr affects p21 functions through a physical interaction. To assess the effect of Vpr on transcription of p21, U-87MG cells were transfected with reporter plasmids containing the various regions of the p21 upstream regulatory sequence fused to the reporter gene along with a plasmid expressing Vpr. As shown in Fig. 1A, Vpr was able to enhance p21 promoter activity, and the minimum DNA sequence spanning between −93 and +1 of the p21 promoter is sufficient for Vpr to exert its activity. Close examination of the DNA sequence spanning −93 to +1 revealed the presence of several characteristic GC-rich motifs, putative binding sites for the Sp1 transcription factor, positioned upstream from the TATA box at position −44. Results from cluster mutants with 10 nucleotide changes across the p21 minimal promoter region suggest the importance of the DNA sequence (nucleotides −84 to −74) in Vpr activation of the p21 promoter (Fig. 1B, lane 2). As anticipated, alterations within the region spanning the TATA box (−54 to −44), where the transcription initiation complex is formed, drastically suppressed transcriptional activity of p21. Further mutations within the region −84 to −74 led us to conclude that the nucleotide sequence (nucleotides −79 to −76) is dispensable for Vpr activation of the p21 promoter (Fig. 1B).

Previously, it has been shown that the region encompassing nucleotides −84 to −74 within the p21 promoter contains a GC-rich motif, putative binding sites for the Sp1 transcription factor (16). Therefore, it was necessary to elucidate the role of Vpr in the presence and absence of Sp1 and to examine whether Vpr binds the responsive DNA sequences directly in order to activate p21 promoter. To that end, we examined the importance of this sequence in transcriptional activation of p21 by Sp1 in astrocytic cells. As shown in Fig. 2A, the mutation within −84 to −74 severely affected the level of p21 activation by Sp1, suggesting a critical role for the distal GC-rich motif in transcription of the p21 gene (Fig. 2A, compare lanes 2 and 5). Co-expression of Sp1 and Vpr significantly elevated the level of transcription from the wild type but not the mutant p21 promoter, pointing to the potential cooperation between Vpr and Sp1 and the importance of the sequence between −84 to −74 for this event (compare lanes 3 and 6).

2 B. E. Sawaya, personal observation.
In an attempt to further demonstrate the functional interaction of Vpr with Sp1, we performed a similar set of transfection assays in an Sp1 null cell line, SL2. As shown in Fig. 2B, expression of Vpr had no effect on the basal transcription of the full-length (−2301 to +1) and the minimal (−93/Si+1) promoters of p21 (Fig. 2B, lanes 1 and 4). Most interestingly, in the presence of Sp1, Vpr gained the ability to augment the activity of the p21 promoter (Fig. 2B, lanes 3 and 6). The observed enhancement of p21 transcription was specific and required the intact distal GC-rich motif (−84 to −74), as the mutant −93-Smut2 showed no significant response to Vpr activation (Fig. 2B, lane 9). These experiments led us to conclude that activation of the p21 promoter by Vpr depends on the presence of Sp1.

Later we examined the ability of Vpr to bind the DNA by bandshift assays using nuclear extracts prepared from Vpr, Sp1-, or Vpr plus Sp1-transfected cells, and a DNA probe spanning sequences between −89 to −70 or its mutant variant. The mutant oligonucleotide had two base substitutions, at positions −79 and −80, and two others deleted at positions −77 and −78 that prevents its association with Sp1 (16). As shown in Fig. 2C, the intensity of the band corresponding to the DNA-Sp1 complex was slightly enhanced in U-87MG cells transfected with a plasmid expressing Sp1 (Fig. 2C, compare complex C1 in lanes 3 and 6). The −89/−70 sequence is the binding site for distinct transcription factors, including members of the Sp1 family (16). To examine whether proteins of the Sp1 family were present in complex C1, we performed supershift experiments by using anti-Sp1 antibody. The presence of the Sp1 protein in complex C1 was demonstrated, as addition of anti-Sp1 antibodies (Fig. 2C, lanes 5 and 10) but not a nonimmune serum (lanes 4 and 9), led to the up-shifting of the complex and the formation of the new complex C0. The Sp1 antibodies were unable to completely abolish the binding of the complex C1 to the DNA, suggesting that in addition to Sp1 other proteins are present in the C1 complex (Fig. 2C, complex C1, lanes 5 and 10). Competition using unlabeled wild type or mutant DNA probes verified the specificity of the complex (data not shown). When the mutant probe was used, the complex C1 was lost, and only the nonspecific complex remained (Fig. 2C, lanes 12–15). Furthermore, the mutant DNA probe failed to bind Sp1 in the absence and presence of Vpr (Fig. 2C, lanes 13–15).

Results from the functional interaction of Vpr and Sp1 provided a rationale to investigate the physical association of these two proteins and to identify the regions within either Sp1 or Vpr, which are important for their cooperativity. A series of N-
and C-terminal deletion mutants of Sp1 were created, and after production by a cell-free translation system, they were used in GST pull-down assay using GST or GST-Vpr. As shown in Fig. 3A, Sp1 contains three zinc finger domains, ZF1, ZF2, and ZF3, which are positioned between the C- and D-domains in the C terminus of the protein. Removal of ZF1 slightly decreased the level of Vpr interaction with Sp1 (Fig. 3B). Further deletion of the protein that removed ZF2 showed an increasingly negative effect on the association of Vpr and Sp1. Deletion of all three zinc fingers abrogated the interaction of Vpr with Sp1. Fig. 3B illustrates representative results from GST pull-down assay. These observations suggest that the zinc finger domains of Sp1 are critical for Vpr association.

Next, we sought to identify the Sp1-binding domain within the Vpr. Previously, we as well as others demonstrated that Vpr-LR, a mutant that lacks the leucine-rich region, was unable to activate HIV-1 LTR (36, 37). Therefore, it was of interest to examine the ability of this mutant to bind to Sp1. To that end, U-87MG cells were transfected with 10 μg of nuclear extracts prepared from cells transfected with either Sp1 or Vpr, or in combination, as indicated above each lane. Supershift experiments were performed with 10 μg of nuclear proteins in the presence of antibodies directed against Sp1 as indicated. C1 demonstrates complexes formed upon addition of nuclear extracts. NS (nonspecific) points to the position of a nonspecific complex, which was detected in some but not all reactions. C0 points at the complex supershifted by Sp1 antibodies. P indicates free probe.

**Fig. 2.** Transcription activation of the p21 promoter by Vpr in the presence of Sp1. A, U-87 MG cells were transfected with 0.5 μg of the reporter p21 luciferase plasmids alone or in combination with 2.5 μg of either Vpr or Sp1 expression plasmids as described in Fig. 1. The data represent the mean value of at least three separate transfection experiments. B, SL2 cells were transfected with 0.5 μg of the p21-luc promoter constructs of either pPac-Sp1 or CMV-Vpr expression plasmids. Empty pCEP-4 or pPac-0 vectors were added in all transfection mixtures to normalize the amounts of the DNA in each reaction. Cell extracts were prepared 48 h after transfection, and luciferase activity was determined. C, bandshift assay was performed by using 32P-labeled, 20-bp double-stranded synthetic DNA fragments containing the wild type GC-rich motif of the p21 promoter (∼89–70) or its mutant with no binding site to Sp1. Approximately 105 cpm labeled DNA probes were incubated with 10 μg of nuclear extracts prepared from cells transfected with either Sp1, Vpr, or in combination, as indicated above each lane. Supershift experiments were performed with 10 μg of nuclear proteins in the presence of antibodies directed against Sp1 as indicated. C1 demonstrates complexes formed upon addition of nuclear extracts. NS (nonspecific) points to the position of a nonspecific complex, which was detected in some but not all reactions. C0 points at the complex supershifted by Sp1 antibodies. P indicates free probe.
Western blot analysis revealed that Sp1 was able to interact with the wild type (Fig. 3C, lane 5) but not with Vpr mutant (lane 6). The interaction between the two proteins was specific, because no interaction was observed with the rabbit serum or with the beads alone. These findings lead to the conclusion that the zinc finger domain within Sp1 and the leucine-rich region within Vpr are necessary for the physical interaction between these two proteins.

To correlate the observed protein-protein interaction data with the functional cooperativity between Sp1 and Vpr on the p21 promoter, U-87MG cells were transfected with the p21 minimal promoter reporter construct (−93-Si/+1) and plasmids expressing Vpr, Vpr-LR, wild type Sp1, and the various Sp1 mutants. Results from transfection assays revealed that complete or partial removal of the A-domain of Sp1 has no significant effect on the ability of Sp1 to stimulate transcription of p21 (Fig. 4A, compare lanes 4 and 6 with lane 2). Deletion of ZF1, ZF2, and ZF3 severely affected the ability of Sp1 to stimulate the activity of the p21 promoter (Fig. 4A, lanes 8, 10, and 12). Expression of the central domain of Sp1 (167−565) showed a modest effect on the basal transcription of p21 (Fig. 4A, lane 16), whereas the mutant 167−350, lacking the zinc finger domain and a portion of the B-domain, failed to exert an activity on p21 (Fig. 4A, lane 14). When the cells were co-transfected with Vpr and Sp1 deletion mutant expression plasmids, only the Sp1 mutant (167−785) was able to cooperate with Vpr (Fig. 4A, lane 5). The level of cooperativity of Vpr with various Sp1 deletion mutants was low in all other cases (Fig. 4A, lanes 7, 9, 11, 13, 15, and 17). These observations suggest that the N terminus of Sp1 between residues 167 and 266 that span the Glu-rich, and the C-terminal region containing the zinc finger domain are essential for full cooperativity with Vpr in stimulating transcription of the p21 promoter. Note that Vpr modestly activates the p21 promoter even in the presence of Sp1 deletion mutants, and this is because these cells contain endogenous Sp1, and Vpr may cooperate with endogenous Sp1.
This effect was not observed in SL2 cells (data not shown).

Later the transfection assay was repeated using p21-Luc (full-length or deletion mutant) alone or in different combinations with Vpr-LR and Sp1 expression plasmids. We found that Vpr-LR failed to activate p21 gene expression either alone or in combination with 2.5 μg of plasmids expressing Vpr-LR and/or Sp1. The amounts of DNA in each transfection mixture were normalized with pCDNA3. Luciferase activity was determined 48 h after transfection. The data represent the mean value of at least three separate transfection experiments.

Induction of p21 promoter by Vpr prompted us to address the physiological significance of such induction. Furthermore, our recent observations demonstrated that HIV-1 LTR activation by Vpr depends on the presence of p21 gene in the cells. Therefore, to gain insight into the mechanism of Vpr stimulation of p21 and the involvement of Sp1 in this event, we performed Western blot assays. U-87MG and SL2 cells were transfected with Vpr and/or Sp1 expression plasmids by using precipitation by the calcium phosphate method. Forty-eight hours after transfection, the cells were washed and lysed, and the nuclear extracts were prepared. As shown in Fig. 5A, using anti-p21 antibodies, we were able to detect p21 in both cell lines (lanes 1 and 5). The endogenous level of p21 was also induced in Sp1-transfected cells (Fig. 5A, lanes 3 and 7). Co-expression of both plasmids, Vpr and Sp1, led to an increase in the endogenous level of p21 slightly higher to the one observed with Sp1 alone (Fig. 5A, compare lanes 4 and 8 with lanes 3 and 7). As a positive control, anti-Sp1 and anti-Vpr antibodies were used to show the efficiency of transfection (Fig. 5A).

Next, we performed a series of transient transfection assays in U-87MG cells using HIV-1 LTR-Luc alone or in the presence of an increasing amount of Vpr (wild type or Vpr-LR) expression plasmids (0.1, 0.25, 0.5, and 1.0 μg, respectively) (Fig. 5B and C). Forty-eight hours post-transfection, the cells were harvested, and half of them were subjected to Western blot assay, whereas luciferase assay was performed on the other half. As anticipated, luciferase assays revealed the activation of HIV-1 LTR by Vpr and that the maximal activation was observed when 0.25 μg of Vpr was used (8.02-fold) (Fig. 5B, lane 3). As mentioned, the other half of the cells were washed and lysed, and the whole cell extracts were prepared. Fifty micrograms of cellular proteins were used for Western blot analysis by using anti-p21 antibody. As shown in Fig. 5B (p21 panel), the endogenous level of p21 was induced in the presence of Vpr. Most

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**Fig. 4.** Functional interaction between Vpr (wild type and mutant) and Sp1 (wild type and mutants). **A,** the effect of Sp1 deletion mutants on the activity of the p21 promoter was studied in the presence and absence of the Vpr plasmid by transient transfection. The values reflect fold induction of the p21 promoter. **B,** U-87MG cells were transfected with 0.5 μg of the various p21-luciferase deletion mutant plasmids either alone or in combination with 2.5 μg of plasmids expressing Vpr-LR and/or Sp1. The amounts of DNA in each transfection mixture were normalized with pCDNA3. Luciferase activity was determined 48 h after transfection. The data represent the mean value of at least three separate transfection experiments.
surprisingly, the maximal induction of p21 correlated with the highest level of HIV-1 gene expression by Vpr (Fig. 5B, lane 3). As expected, Vpr-LR failed to activate the HIV-1 promoter (Fig. 5C, lanes 2–5). No change in p21 endogenous level was observed in the Vpr-LR-transfected cells (Fig. 5C). To control the equal loading of proteins, anti-/H9252-actin antibody was used (Fig. 5, B and C, actin panels). Our results clearly showed the existence of a functional interplay between Vpr and Sp1 in affecting the level of p21 and modulating p21 gene expression. These results confirmed our previously observed data in which we showed that induction of HIV-1 LTR by Vpr is p21-dependent.2

Previously, it has been shown that in the absence of its potential binding site, the tumor suppressor protein, p53, might activate the p21 promoter through its interaction to the region spanning nucleotides −84 to −74 (46). Furthermore, we previously demonstrated the existence of a functional interplay of Vpr, Sp1 with p53 which can dictate the level of HIV-1 LTR transcription (36). In light of these observations, we performed a series of transfection experiments to assess the effect of p53 on the functional cooperation of Sp1 and Vpr on transcription of p21. In the first series of experiments, we utilized the full-length p21 promoter reporter construct (−2301 to +1). As expected, p53 elevated the level of p21 gene expression in these cells (Fig. 6A, lane 3). Moreover, p53 was able to augment the level of activation of p21 by Sp1 (Fig. 6A, compare lane 1 with lane 5) and Sp1 plus Vpr (compare lane 4 with lane 7) but not Vpr alone (compare lane 2 with lane 6). To test whether the observed positive impact by p53 requires the GC-rich motif located within −84 to −74, a similar series of transfection assays were performed by using the p21 minimal promoter constructs. As expected, the results verified the ability of p53 to amplify the effect of Sp1, and Sp1 plus Vpr, but not Vpr alone on p21 promoter transcription. The stimulatory effect by p53 was not observed when mutant promoter constructs were used, suggesting that p53 can enhance transcription of the p21 gene by recruiting Sp1 and Vpr.

We then examined the ability of the p53 protein to affect

FIG. 5. Physiological significance of the induction of p21 by Vpr and/or Sp1. A, 100 μg of nuclear proteins extracted from U-87MG and SL2 cells transfected with different expression plasmids were used to detect p21 using anti-p21 antibodies. Anti-p21, anti-Sp1, and anti-Vpr were used to indicate the position of p21, Sp1, and Vpr, respectively. B and C, increasing amounts of Vpr or Vpr-LR (0.1–1.0 μg) were transfected in combination with 0.5 μg of the LTR-luciferase reporter construct U-87MG cells. Forty-eight hours post-transfection, the cells were washed and divided into two parts. Luciferase assay was performed on one-half where luciferase activity was determined (S.E. ± 18%). Western blot analysis was performed by using the whole cell extracts isolated from the other half. Anti-p21 and/or anti-/H9252-actin antibodies reveal the variations in the endogenous level of p21 or the equal loading of proteins, respectively.
DNA binding activity of Sp1 in the presence and absence of Vpr by electrophoretic mobility shift assay. Nuclear extracts were isolated from cells transfected with different combinations of Sp1, Vpr, and p53 expression plasmids. Results from bandshift experiments revealed that the addition of Vpr, p53, and Sp1, separately or in different combinations to the binding reaction, elevated binding of Sp1 to the DNA probe spanning nucleotides -84 to -74 of the p21 promoter sequence (Fig. 6B, compare lanes 1 with lanes 2–4 and 8–11). Most interestingly, the complexes formed with Vpr, Sp1 and/or with p53 co-migrate almost at the same position (Fig. 6B, compare lane 1 with lanes 2–4 and 8–11). The use of anti-Sp1 (Fig. 6B, lanes 5 and 12) or anti-p53 (lanes 6 and 13) antibodies showed the existence of Sp1 and/or p53 proteins in the complex C1, and the formation of new complex C0 (lanes 5 and 6). The specificity of the protein-DNA complex formation was confirmed with the use of preimmune rabbit serum (Fig. 6B, lanes 7 and 14).

The existence of a functional interaction between Vpr, Sp1, and p53 prompted us to examine the ability of the three proteins to interact with each other in vitro as well as in vivo. In
the next series of experiments, we assessed the binding of p53 to Vpr or to Sp1 either alone or together by GST pull-down assay. The experiment was performed using in vitro synthesized p53, Vpr, and Sp1. As shown in Fig. 7, we obtained no binding activity pointing to direct interaction of p53 with Vpr (Fig. 7, A and B). Conversely, Sp1 was retained by GST-p53 and not GST indicating its ability to associate with p53 (Fig. 7C). Most interestingly, when Sp1 was present, a band corresponding to Vpr was detected upon elution of GST-p53 but not GST (Fig. 7D). This observation suggests that the interaction of Sp1 and p53 may alter the configuration of p53, allowing association of p53 with Vpr.

To confirm these observations further, we performed a series of IP/Western assays by using extracts isolated from SL2 cells. The cells were transfected with 10 μg of p53, Sp1, and/or Vpr-FLAG expression plasmids separately or by using different combinations. Forty-eight hours post-transfection, the cells were harvested, and the whole cell extracts were prepared. Fifty and 300 μg of protein were used for Western analysis or IP, respectively. As shown in Fig. 7E, and as expected, an endogenous level of p53 was induced in p53-transfected cells (compare lanes 1 and 2). Immunoprecipitation assays revealed the existence of a physical interaction between Sp1 and p53 in the presence and absence of transfected Vpr (Fig. 7E, compare lanes 3 and 6). p53 was also able to interact with Vpr in the presence of Sp1 but not in its absence (Fig. 7E, compare lanes 4 and 7). The same experiments were repeated using anti-Sp1 (Fig. 7F) or anti-FLAG (Fig. 7G) antibodies, respectively. The existence of an interaction between Vpr and p53 was only possible in the presence of Sp1. Rabbit serum was used as a control to show the specificity of such interaction (Fig. 7, E−G, lanes 8).

To demonstrate further functional cooperativity between p53, Sp1, and Vpr, we utilized a synthetic promoter reporter construct containing six classical Sp1-binding sites (6xSp1-CAT) fused to a minimal promoter sequence from HIV-1 (47). As anticipated, in the presence of Sp1, the level of transcription from the synthetic promoter was increased (Fig. 7H). In the presence of p53, the level of activation of the promoter by Sp1 was slightly improved. Co-expression of Vpr and p53 showed a minimal, if any, effect on the transcription of the synthetic promoter. Combined production of Vpr, p53, and Sp1, however, caused a drastic effect on the transcriptional activation of the test promoter, suggesting that the observed physical interaction of Vpr and p53 in the presence of Sp1 may have a functional consequence on the promoter, which is responsive to Sp1 activation.

Finally, to elucidate the role of full-length Vpr and its functional interplay with p53, and Sp1, we utilized the plasmids expressing either Vpr mutant (Vpr-LR) and/or HIV-1 Tat. We performed a series of transfection assays using Vpr-LR, p53, Sp1, and Tat expression plasmids in different combinations. In the first series of experiments, we utilized the full-length p21 promoter reporter construct (−2301 to +1). Neither Vpr-LR nor Tat affected the level of p21 gene expression in these cells (Fig. 7I, lanes 1 and 2). Moreover, the ability of p53 to augment the level of activation of p21 by Sp1 was not affected in the presence of Vpr-LR or Tat (Fig. 7I, lanes 3 and 4). Similar results were obtained when the minimal p21 promoter construct (−93/S+1) was used (Fig. 7I, lanes 5−8). These results demonstrate that the LR region within Vpr is important for the regulation of p21 gene expression. In addition, although HIV-1 Tat has the ability to activate HIV-1 LTR as well as other cellular promoters, Vpr remains the only known HIV-1 activator of p21 gene expression.

**DISCUSSION**

In this study, we identified the mechanisms used by Vpr to regulate p21 gene expression and the involvement of p21 and Vpr in the regulation of HIV-1 LTR. We have further documented that a specific DNA sequence located between −84 and −74 of the p21 promoter is responsive to Vpr activation. This DNA motif possesses binding affinity to Sp1 and Vpr without being physically associated with the Sp1-DNA complex and enhances binding of Sp1 to this sequence, indicating that activation of the p21 promoter by Vpr requires the presence of Sp1. In the Sp1 null cells, transcription of p21 remained unchanged in the presence of Vpr.

The functional interplay observed between Vpr and Sp1 prompted us to investigate potential physical interactions between these two factors. Our analyses showed that Vpr interacts functionally and physically with Sp1 and that their interactions require the leucine-rich region within Vpr, and at least one of the two glutamine domains and/or the DNA binding domain of Sp1, whereas the other domains are dispensable. The involvement of the zinc finger DNA binding domain of Sp1 in physical interactions with other proteins is not unprecedented. For example, Sp1 was shown to interact physically via its DNA binding domain with transcriptional activators such as the p65/RelA subunit of NF-κB (31), the erythroid factor GATA-1 (48), YY1 (49), the cell cycle regulator E2F (29), and c-Jun (15). In all of the above cases, physical interactions were associated with functional cooperation among the partners on target promoters that contain the closely spaced putative binding sequences for the two proteins. In the case of the p21 promoter, the synergistic mechanism of transactivation by Vpr and Sp1 is distinct from the above-mentioned examples. First, Vpr cannot bind to the p21 promoter, and thus functional cooperation could not be the result of a juxtaposition of the two proteins on the DNA. Second, the glutamine domain of Sp1 is sufficient for the superactivation of Sp1 by Vpr, whereas this domain is dispensable for the interaction of Sp1 with all of previously identified proteins. Finally, our findings establish that the association of Vpr with Sp1 can occur in the absence of DNA. However, in the presence of DNA, the interaction of the two proteins results in enhanced DNA binding of Sp1 to its cognate site. This phenomenon has also been observed previously in the cases of Rh/Sp1 (50), SREBP-1/Sp1 interactions (51), and c-Jun/Sp1 (15). Thus, we are tempted to speculate that the association of Vpr with Sp1 results in a conformational change in the Sp1 molecule, which can lead to enhanced DNA-binding properties. We could also hypothesize that specificity in Sp1 function could result from the different configurations that Sp1 could adopt as a result of its interaction with different transcription factors. Finally, it is of interest that the Gln-rich region of Sp1 has also been shown to be required for the transcriptional induction of Sp1-dependent promoters by transforming growth factor-β (52). In their observations, the authors suggest that in HEPG2 cells, c-Jun and Smad proteins regulate Sp1-dependent transcription via a similar mechanism (52).

U-87MG cells constitutively express functional p53, as reported previously (53). It has been shown that the p53-mediated transactivation of the p21 promoter was lost by deletion of the distal p21 promoter region or the proximal Sp1-binding sites. These findings strengthen the hypothesis that synergism between p53 and Sp1 occurs on the p21 promoter, which correlates with previous findings (46). Mutagenesis analysis showed that the Sp1-binding site 3, located between nucleotides −82/−77, is essential for the p53-mediated synergistic transactivation of the p21 promoter. In contrast, mutations in the other Sp1-binding sites showed no significant increase in
FIG. 7. Physical and functional interplay among Vpr, Sp1, and p53. A–D, in vitro synthesized 35S-labeled p53, Vpr, and/or Sp1 were incubated with GST, GST-Vpr, or GST-p53 as indicated above each panel. Bindings of the proteins to GST or to the GST fusion protein are marked by an arrow. E–G, 300 μg of whole cell extract isolated from SL2 cells transfected with different combinations of Vpr, p53, and/or Sp1 were used for IP using anti-Sp1, anti-p53, or anti-FLAG antibodies as indicated for each panel. Western analysis confirmed the interaction between the three proteins and the formation of a ternary complex. Rabbit serum was used as a negative control. H, the cells were co-transfected with 1.0 μg of the reporter plasmid containing 6 Sp1-binding sites at the 5'-end of the CAT DNA sequence and 1.0 μg of plasmids expressing Vpr, p53, and/or Sp1. CAT activity was determined after 48 h and presented as fold activation. The basal level of the 6xSp1-CAT transcription was set at 1.0. I, U-87MG cells were transfected with 0.5 μg of the various p21-luciferase deletion mutant plasmids either alone or in combination with 2.5 μg of plasmids expressing Vpr-LR, Tat, p53, and/or Sp1. The amounts of DNA in each transfection mixture were normalized with pCDNA3. Luciferase activity was determined 48 h after transfection. The data represent the mean value of at least three separate transfection experiments. The fold activation is represented above each lane.
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p53-mediated transactivation of the p21 promoter. These findings strongly indicate that p53-mediated transactivation of the p21 promoter is achieved by the functional cooperation between p53 and Sp1 via the Sp1-binding site 3. Indeed, the interaction of Sp1 with the other GC-rich motifs located in the proximal region may modulate the overall activity of the p21 promoter.

The Sp1-binding site 3 has been shown previously to mediate p21 induction by various agents such as transforming growth factor β (54), butyrate (55), the histone deacetylase inhibitor trichostatin A (56), and Ca2⁺ (57) among others. In contrast, Sp1 sites 1 and 2 mediate transcriptional activation by phorbol esters and okadaic acid (58), and the gut-enriched Kruppel-like factor (GKLF and KLF4) (59). Thus far, no specific role has been attributed to the most proximal and overlapping Sp1 sites 5 and 6. All the above observations suggest that utilization of the Sp1-binding site under different conditions, is an important event for p21 regulation. The mechanism underlying this specificity is currently unknown. It is possible that the distance of the individual Sp1-binding sites from the TATA box of the p21 promoter that serves as a point of assembly of the factors of the transcription machinery determines the identity of the activators or co-activators that interact with each site, and this may determine its contribution to p21 promoter levels under constitutive or inducible conditions.

The studies presented in this communication are in accord with earlier observations (36) on the cooperative interaction of Vpr with Sp1 and p53 and its effect on p21 gene transcription. Although some of the mechanisms involved in such interactions remain unclear, activation of p21 gene transcription by Vpr is intriguing in light of the findings that this small protein incorporates into the virion and enters into the newly infected cells by associating with the capsid proteins. It is possible that added Vpr and not Tat in the newly infected cells stimulates transcription of the viral genome at the immediate early phase, by using cellular genes such as p21, and leads to rapid production of other regulatory proteins such as Tat and Rev that are pivotal for productive viral lytic infection.

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Interplay between HIV-1 Vpr and Sp1 Modulates p21WAF1 Gene Expression in Human Astrocytes
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