Cooperative Actions of HIV-1 Vpr and p53 Modulate Viral Gene Transcription*

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Transcription of the human immunodeficiency virus type-1 (HIV-1) genome is controlled by cooperative interaction of viral encoded proteins and host regulatory proteins. In this study, we have examined the capacity of the viral accessory protein, Vpr, to modulate transcriptional activity of the HIV-1 promoter sequence located within the long terminal repeat (LTR). We demonstrate that ectopic expression of Vpr in human astrocytic cells, U-87MG, enhances the basal activity of the viral promoter in transfected cells and that the GC-rich sequences, spanning nucleotides −80 to −43, are important for this activity. Since this region serves as the target for p53-induced suppression of LTR activity and interacts with the ubiquitous transcription factor, Sp1, we examined the cooperative activity of Vpr, p53, and Sp1 upon LTR transcription. Results from co-transfection studies indicated that overexpression of wild type p53, but not mutant p53, decreases the level of activation of the LTR by Vpr. Transcriptional activation of the LTR by Vpr required the presence of Sp1 since overexpression of Vpr in cells with no endogenous Sp1 failed to augment LTR activity. Results from protein-protein interaction studies indicated that Vpr is associated with both p53 and Sp1 in cells with ectopic expression of these proteins. Moreover, it was evident that p53 and Sp1 interact with each other in these cells. These functional and structural studies provided a working model on the cooperative interaction of Vpr with cellular proteins Sp1 and p53 and control of viral gene transcription at immediate early stage of infection prior to the participation of other viral regulatory proteins.

The genome of the human immunodeficiency virus type 1 (HIV-1) contains, in addition to common retroviral genes encoding structural proteins gag, pol, and env, several auxiliary genes with a different degree of importance for replication of the viral genome. While the auxiliary genes responsible for production of Tat and Rev are essential for viral gene expression and replication, the remaining group of this family, so-called accessory genes which include vpr, vpu, nef, and vif, are dispensable for virus replication in cell culture (1). Mutagenesis studies, however, have indicated that any alterations in these genes may have an impact upon the replication properties of HIV-1.

Vpr, the 96-amino acid long viral protein, represents one of the most studied accessory proteins. This small protein is of particular interest among the other HIV-1 accessory proteins because of its association with the virus particles and its unique structural feature (2–4). Results from biochemical and genetic studies have revealed several interesting properties of this protein. For example, in infected cells, Vpr is localized, accumulates in nuclei (5, 6), and has the ability to interact with several cellular regulatory proteins (6). This protein can arrest cells at the G2 stage of the cell cycle by affecting cdc 2/cyclin B expression and activity (7–13). In addition, earlier studies have indicated that Vpr has the ability to augment transcription of the HIV-1 LTR in cell-free in vitro transcription assay (14). These observations led to the assumption that the envelop- incorporated Vpr, by up-regulating viral gene expression in newly infected cells at the immediate early phase, increases expression of other viral genes including the potent transcription transactivator, such as Tat, in the subsequent lytic cycle. Whereas the mechanism whereby Tat exerts its regulatory action upon virus gene transcription is greatly understood (for review, see Ref. 15), the pathway by which Vpr induces promoter activity of HIV-1 remains elusive. In previous studies, results from in vitro transcription assay suggested that Vpr may induce transcription of the HIV-1 promoter through the GC-rich motif of the LTR (14), which serves as the binding site for the ubiquitous cellular transcription factor, Sp1 (16), and that interaction of Vpr and Sp1 may be important for the observed activity (14). Moreover, results from protein-protein studies indicated in vitro association of Vpr and TFIIB (17). These observations demonstrate that high level expression of the viral genome may require cooperative interaction of several viral proteins, such as Tat, Vpr, and the cellular proteins that may share a common target motif within the viral genome. The ability of Vpr to stimulate the HIV-1 promoter through the GC-rich motif, a region which has been demonstrated to negatively respond to the p53 tumor suppressor protein, prompted us to launch a series of functional and structural studies to assess the ability of Vpr to regulate HIV-1 promoter activity in cell culture and to determine the importance of the GC-rich binding protein and p53 in this regulatory event.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The plasmid constructs containing various sequences of the HIV-1 LTR upstream of the reporter CAT gene have been described previously (16). The reporter constructs with an internal deletion in the GC-rich motif were provided by Dr. S. Zeichner NCI, National Institutes of Health, (Bethesda, MD) (19). The pCDNA Vpr expression plasmid containing the CMV promoter expressing Vpr was provided by Dr. A. Srinivasan (Thomas Jefferson University, Philadelphia, PA) and has been described previously (20). The Sp1 expression vector was generously provided by Dr. R. J. Tjian (University of Cali-
RESULTS AND DISCUSSION

Vpr is a 96-amino acid protein encoded by the HIV-1 genome that has the ability to increase the rate of replication of the virus in T-cell lines and peripheral blood mononuclear cells (PBMCs) (32, 33). Earlier studies have indicated that Vpr has a transcriptional activity and induces expression of the viral LTR and several cellular genes (34). While activation of the viral promoter by Vpr may be mediated by TFIIIB, a component of the transcription initiation complex (17), earlier studies pointed to the importance of the GC-rich motif located upstream of the transcription start site, in the observed activation of the HIV-1 gene by this protein. Here, we performed transfection studies utilizing full-length and deletion promoter constructs derived from the HIV-1 regulatory region to evaluate the ability of Vpr in enhancing the activity of HIV-1 LTR in the human astrocytic cell line, U-87MG. Results from this study indicated that ectopic expression of Vpr in U-87MG cells transfected with the reporter plasmid containing the LTR promoter sequences from -458 to +80, -117 to +80, and -80 to +80 results in 6.4–7.0-fold activation of the viral promoter (Fig. 1). Removal of the sequences between −80 to −48, that deletes the GC-rich motif, completely abrogated Vpr responsiveness and LTR activation in these cells. The use of 3′ deletion constructs that remove the sequences between +80 to +3 suggested that the sequences positioned downstream from the transcription start site (+1) may not play an important role in Vpr-induced activation of the LTR in these cells (Fig. 1). Results from studies utilizing internal deletion constructs of the LTR that remove the sequences between −80 to −48 from the full-length (3 and 7), and the 5′ deletion constructs (5 and 9) as depicted in Fig. 1, in the transfection experiments verified that the sequences between −43 to −80 are essential for induction of LTR transcription by Vpr in astrocytic cells.

In earlier studies, we and others (35–37) demonstrated that the p53 tumor suppressor gene product has the ability to decrease transcriptional activity of the HIV-1 LTR in cell culture and in a cell-free transcription system and that the sequences spanning the GC-rich motif of the LTR are important for this activity (35). The ability of Vpr to stimulate transcription of the LTR through the GC-rich sequences prompted us to examine the potential cooperative action of Vpr and p53 on transcriptional activity of the HIV-1 promoter. In the first series of
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Studies revealed that treatment of the cells with dexamethasone decreased basal and Vpr-mediated transcriptional activity of the viral promoter and that the maximum level of inhibition was achieved at 18 h when the level of p53 is at the highest (Fig. 3, B and C). It should be noted that treatment of the cells with dexamethasone had no significant effect on the basal transcription of the HIV-1 LTR in these cells. These observations, along with the previous co-transfection data, strongly suggest that functional interaction between Vpr and p53 may modulate the level of Vpr transcriptional activation upon the LTR promoter.

As described previously, p53 has been shown to act as a transcriptional activator and repressor in a variety of cells. To examine the effect of Vpr in the activation of the p53 responsive promoter, we utilized MCK-CAT, which contains the p53 responsive element, and its transcriptional activity is increased by the wild-type p53 (39). In this study, cells were transfected with the reporter MCK-CAT constructs and the wild-type p53 expression plasmid in the absence and presence of the Vpr expression plasmid. As shown in Fig. 4, a substantial increase in the transcriptional activity of MCK promoter was obtained in cells expressing wild-type p53. Co-expression of wild-type p53 and Vpr decreased the level of p53 activation of the MCK promoter in the transfected cells. Of note, expression of Vpr alone in these cells resulted in a modest (2-fold) induction of the MCK promoter. This activation may be attributed to the presence of a GC-rich sequence which resides within the MCK promoter (40). Co-expression of mutant p53 and Vpr in the cells showed a similar effect on the MCK promoter as that observed in cells expressing Vpr alone. These data further indicate that functional interaction between p53 and Vpr may not be restricted to the LTR promoter as evidenced by the cooperative action of these two proteins upon the gene that is positively regulated by p53. The finding that these two proteins in combination exert a negative effect upon LTR activity is reminiscent of the interplay between p53 and Sp1 in activation of HIV-1-LTR (41), where the influence on promoter activity was attributed to alteration in the interaction of Sp1 with DNA by p53.

Since both p53 and Vpr exerted their regulatory action on the HIV-1 LTR through the GC-rich motif, a region which is the target for binding of the ubiquitous transcription factor, Sp1, in the next series of studies we examined the involvement of Sp1 in the observed regulatory event. In this study, SL2 cells, which have no endogenous Sp1, were transfected with LTR-CAT alone or in various combinations with expression plasmids that produce Vpr, wild-type p53, and Sp1. Results from these studies indicated that in contrast to the data obtained from the U-87MG cells (Figs. 3 and 5A), ectopic expression of Vpr and p53 in these cells exhibits no significant effect on LTR promoter activity (Fig. 5B). However, once the plasmid expressing Sp1 was included in the transfection mixture, the level of LTR activity was drastically elevated. Co-production of Vpr and Sp1 resulted in transcriptional activation of the LTR to a level that is comparable with that seen upon expression of Sp1 alone in these cells. Of interest, expression of p53 significantly decreased the level of transcriptional activation of the LTR by Sp1 and Sp1 plus Vpr. These observations suggest that activation of the LTR by Sp1 may be modulated by p53 and that Sp1 function can be counter prevented by p53. As mentioned earlier, these findings corroborate with previous observations indicating that p53 has the ability to alter the DNA binding ability of Sp1 (41). Also, it is evident that Vpr is incapable of augmenting LTR transcription in the absence of Sp1. Further,
In the next series of experiments, we investigated the interaction of Vpr with Sp1 and p53. Initially, U-87MG cells were transfected with p53 and Vpr expression plasmids alone or in combination. In this study, we utilized the fusion Vpr expression plasmid, pCMV-Vpr-flag, which expresses Vpr fused to amino acid flag sequences at the C-terminal region (23). After 36 h, protein extracts were prepared and reacted with either anti-Sp1 antibody, or anti-flag antibody that recognizes Vpr fusion protein. The immunocomplexes were separated from the remaining protein by centrifugation and analyzed by Western blot technique directed for the detection of p53. As shown in Fig. 6A, the anti-Sp1 immunocomplexes from untransfected cells and cells transfected with pCMV-p53 contained p53 protein, suggesting that p53 and Sp1 may be associated with each other in these cells. Of note, the intensity of the band corresponding to p53 in the transfected cell extract was higher compared with the control, indicative of overproduction of p53 by pCMV-p53 (Fig. 6A, compare lane 2 to 1). This observation is in agreement with previous data pointing to the interaction of endogenous Sp1 and p53 (36). The anti-flag-directed immunocomplex obtained from the Vpr transfected cells showed an extremely weak signal corresponding to the p53 band. In contrast, the immunocomplex obtained from the cells with ectopic expression of Vpr and p53 by using anti-flag antibody showed an intense p53 band, suggesting the in vivo association of p53 and Vpr in these cells (Fig. 6A, lane 5). Similarly, protein extract from cells transfected with Vpr and p53 expression plasmids showed the presence of p53 in the immunocomplex that was pulled-down by anti-Sp1 antibody (Fig. 6A, lane 6). Fig. 6B shows that the Sp1-associated immunocomplex from cells transfected with Vpr contains the 15-kDa chimeric Vpr protein. These observations strongly suggest that the HIV-1 regulatory protein, Vpr, is in complex with cellular proteins Sp1 and p53. Of note, results from Western blot analysis revealed an approximately 3-fold increase in the level of Sp1 in the cells transfected with the Sp1 expression plasmid than that of the untransfected cells (data not shown). The endogenous level of p53 in untransfected cells was almost undetectable (Fig. 6C, lane 1). In the extract from the transfected cells, an intense band corresponding to exogenously produced p53 was easily detected (Fig. 6C, lane 2). Furthermore, from the intensity of the bands, it was evident that only 30% of the protein was immunoprecipitated and found in the pellet, whereas 70% of the overproduced p53 remained in the supernatant (Fig. 6C, lanes 3 and 4, respectively). Results from silver staining of the protein gel showed the presence of p53, the immunoglobulin subunits, and one peptide with lower mobility than p53 (data not shown). In the next series of studies, we utilized SL2 cells to investigate the importance of Sp1 in the association of Vpr and p53. These cells were transfected with the expression plasmids alone or in various combinations allowing for ectopic production of Sp1, p53, and Vpr. Of note, SL2 cells contain endogenous p53 protein, which co-migrates with the p53 produced by the expression plasmid (Fig. 6D, compare lanes 1 and 2). This band was not detected in the immunocomplex pulled down with the control preimmune sera (data not shown). Results of the immunoprecipitation/Western blot analyses indicate that the anti-flag-mediated pulled down immunocomplex from cell extracts transfected with Vpr and p53 contains no strong band corresponding to the 53-kDa protein (Fig. 6E, lane 1). Of interest, ectopic expression of Sp1 in the transfected cells resulted in the appearance of a p53 band in the immunocomplex pulled down by anti-flag antibody (Fig. 6E, lane 4). These observations suggest that Sp1 may mediate complexation of
The immunocomplex pulled down by Sp1 from the extract transfected with pCMV-Sp1 alone (lane 2) or pCMV-Sp1 plus pCMV-p53 (lane 3), pCMV-Sp1 plus pCMV-p53, and pCMV-Vpr-flag (lane 4) contains a p53 band, further suggesting the association of Sp1 and p53 in these protein extracts. The studies presented in this communication provide evidence for the cooperative interaction of viral (Vpr) and cellular (p53 and Sp1) regulatory proteins and their effect on HIV-1 gene transcription. Although the mechanism of these observations remains unclear, activation of viral gene transcription by Vpr is intriguing in light of the findings that this small protein, by associating with the capsid proteins, incorporates into the virion and enters into the newly infected cells. As such, it is possible that input Vpr in the newly infected cells stimulates transcription of the viral genome at the immediate early phase and leads to rapid production of other regulatory proteins such as Tat and Rev that are pivotal for productive viral lytic infection. Results from deletion mutant analysis corroborate with earlier results and indicate that the GC-rich nucleotide sequence positioned between nucleotides 280 to 243, which is the binding site for Sp1, is the target for Vpr activation. We also demonstrated that in cells expressing Vpr, Sp1, and p53, Vpr can form a complex with Sp1. This is an interesting observation since in previous studies it was demonstrated that in vitro association of highly purified Vpr and Sp1 requires the presence of at least two Sp1 DNA motifs. Thus, one can envision a model in which stable complexation of Sp1 and Vpr can occur in the absence of Sp1-DNA motifs once the cellular protein, p53, is present. Therefore, p53 may function as a co-factor and facilitate interaction of Vpr and Sp1. Experiments are currently in progress to establish functional relevance of such interactions through the use of mutant versions of these proteins that lack the ability to interact with each other. It is also interesting to remember that p53 is a major player of host cell cycle machinery, and by modulating a series of cellular genes such as p21, can dictate the rate of G1, G1/S, S, and G2 phases.

**FIG. 5.** Interplay between Vpr and p53 in the absence of Sp1 protein. A, the activity of the Flag-tagged Vpr was examined in U-87MG and compared with that of Vpr. Transfections were performed as described in Fig. 1. B. Drosophila Schneider SL2 cells that are deficient in endogenous Sp1 were used in these studies. Five hundred nanograms of the LTR-CAT promoter construct were co-transfected together with either empty pPac-0 plasmid or 5 μg each of the pPac-Sp1, pPac-p53, or CMV-Vpr expression plasmids. Cell extracts were prepared 48 h posttransfection and were subjected to CAT analysis. Histograms show the fold promoter activation assessed by CAT activity expressed relative to the value obtained with the reporter construct alone. Standard deviations did not exceed 15%.

**FIG. 6.** Association of Vpr and p53 in vivo. Cell lysates (300 μg) were prepared from U-87MG (panels A–C) or Drosophila SL2 cells (panels D and E) following transfection with various expression plasmids, alone or in combination, as indicated. Immunoprecipitations (designated as IP) were performed using specific antibodies that detect Sp1, p53 (pAb 421), or flag peptide. Immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot. The antibodies used for immunoprecipitation are indicated at the bottom of the panels. Panel B represents the levels of Vpr immunoprecipitated with either flag (lane 1) or Sp1 (lane 2) antibody. In panel C, lanes 1–4 represent endogenous, exogenously expressed, immunoprecipitated p53, and the amount remaining in the supernatant, respectively, following Western blot analysis using p53 antibody. Panel D represents the endogenous (lane 1) and exogenous (lane 2) levels of p53 in Schneider cells (SL2) as determined by immunoprecipitation.
of the cell cycle (42). Also, earlier studies by several laboratories have indicated that Vpr can induce cell cycle arrest of the cells in the G1 phase by preventing activation of the mitotic cyclin-dependent kinases and thereby prevent cells from undergoing mitosis and proliferation (for review, see Ref. 43). Although, the significance of these observations with respect to HIV-1 gene transcription and replication remains to be established, it is noteworthy to mention that cooperative interaction between another viral protein, Tat, and host-cell cycle machinery results in elongation of the G1 phase and prevents rapid cell proliferation (14, 44). Thus, it appears that both HIV-1 regulatory proteins, Vpr and Tat, can stall cell cycle proliferation on one hand, and stimulate viral gene transcription on the other hand, evidently, to exert their dual activity on viral and host functions, these regulatory proteins require interaction with cellular proteins such as Sp1 and p53 (for Vpr) as presented in this communication, and NFκB and pRB for Tat (45, 46). As such, our future efforts will rest on better understanding of the cooperative interaction of viral and host regulatory proteins and their effect on viral and host function.

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