The HIV-1 Vpr co-activator induces a conformational change in TFIIB

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Abstract Vpr is a HIV-1 virion-associated protein which plays a role in viral replication and in transcription and cell proliferation. We have previously reported that Vpr stimulates transcription of genes lacking a common DNA target sequence likely through its ability to interact with TFIIB. However, the molecular mechanism of the Vpr-mediated transcription remains to be precisely defined. In this in vitro study, we show that the binding site of Vpr in TFIIB overlaps the domain of TFIIB which is engaged in the intramolecular bridge between the N- and C-terminus of TFIIB, highly suggesting that binding of Vpr may induce a change in the conformation of TFIIB. Indeed, with a partial proteolysis assay using V8 protease, we demonstrate that Vpr has the ability to change the conformation of TFIIB. We investigated in this partial proteolysis assay a series of Vpr-mutated proteins previously defined for their transactivation properties. Our data show a correlation between the ability of Vpr-mutated proteins to stimulate transcription and their ability to induce a conformational change in TFIIB, indicating a functional relevance of the Vpr-TFIIB interaction.

Key words: HIV-1 Vpr; TFIIB; Transactivation; Conformation change

1. Introduction

The HIV-1 genome encodes, in addition to *gag*, *pol* and *env* structural gene products common to all retroviruses, accessory gene products which are dispensable for in vitro viral replication but are believed to be important in optimizing the viral life cycle in vivo (for a review, see [1]). Among them, the Vpr protein, which is encoded by an open reading frame located in the central part of the viral genome, is of particular interest. It is one of the HIV-1 non-structural proteins that is incorporated into the viral particles in molar concentrations, strongly suggesting a role in the immediate early events upon infection of permissive cells [2]. In infected cells, Vpr accumulates in the nuclei. Results from a number of studies have revealed that Vpr has several properties. Vpr has been shown to be important in the viral life cycle in facilitating nuclear import of the HIV-1 DNA in newly infected cells, thus allowing infection of non-dividing macrophages [3–7]. Expression of Vpr results in arrest of the cell cycle in the G2/M phase [8–11], induces terminal differentiation in some cell lines [12] and enhances viral replication through its transcriptional properties [13].

We have previously shown that Vpr co-operates with different heterologous promoter-bound activator domains to stimulate transcription [14]. These results, in addition with those from Cohen et al. [13], have demonstrated that the transactivation property of Vpr does not seem to depend on specific responsive promoter sequences. Other studies have indicated that Vpr interacts with the ubiquitous cellular transcription factor Sp1 [15] or with the glucocorticoid receptor [16,17]. Moreover, we have demonstrated that Vpr specifically binds to the general transcription factor TFIIB, indicating that Vpr functions as a transcriptional co-activator [14]. These data, confirmed recently by Kino et al. [17], suggest that the Vpr-mediated transcription requires interactions with specific transcription factors and/or with the basal transcription machinery.

Although Vpr has a transcriptional effect on several promoters, the molecular basis for this activity is unclear. In this study, we investigate the functional relevance of the Vpr-TFIIB interaction. Our data led us to propose a molecular mechanism by which Vpr may function as a co-activator of several heterologous promoters.

2. Materials and methods

2.1. Plasmid constructions

The NL43 Vpr wild-type expression plasmid was used as a template to generate mutated Vpr sequences using a two-step recombinant PCR methodology. PCR-amplified products were then cloned after appropriate enzymatic digestion between *Bam*HI and *Not*I restriction sites of the pGEX-5X-2 plasmid (Pharmacia). GST-Vpr derivatives were named E25K, A30F, V57L and R80A. GST-TFIIB expression plasmid (a kind gift of E. Manet) was used to generate GST-TFIIB derivatives by PCR-assisted in vitro mutagenesis. GST-TFIIB derivatives were named W52A, F52A, W52A/F55A, R53A/T54A and E51A/S65A. GST and GST-Vpr open reading frames have been previously described [14] and were subcloned between *Nco*I and *Xho*I restriction sites into the Pos7 expression vector (kindly provided by B. Moss) and expressed in HeLa cells under the control of the T7 polymerase promoter.

2.2. Analysis of Vpr-TFIIB complexes in cells

To overexpress GST or GST-Vpr, HeLa cells previously infected for 1 h with three plaque forming units of recombinant vaccinia virus (T7-VTF, Ankara strain: a kind gift of G. Sutter) per cell, were transfected with GST or GST-Vpr genes cloned into the Pos7 expression vector. Cells overexpressing GST or GST-Vpr were harvested 24 h after transfection and lysed by freeze-thawed cycles in a buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5% of NP-40 in the presence of an anti-protease cocktail. Expression of the GST derivatives in cell lysates was detected by Western blotting with anti-GST antibody (Santa Cruz Biotechnologies). Expression of Vpr fused to GST was detected in cell lysates by Western blotting with our anti-Vpr antibody obtained from rabbits injected with purified MBP-Vpr fusion protein. Endogenous expression of TFIIB in cell lysates was detected by Western blotting with anti-TFIIB antibody (Santa Cruz Biotechnologies). Upon 30 min incubation on ice, cell lysate was diluted five times with the same buffer

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without NP-40 and incubated for 2 h before addition of GSH-agarose beads. Beads were then recovered by centrifugation and after extensive washes, bound proteins were resolved by SDS-PAGE followed by Western blotting analysis with anti-TFIIB antibody.

2.3. In vitro protein-protein binding assays

GST fusion protein expression plasmids were grown in the *Escherichia coli* TG1 strain. Transformed bacteria were induced with isopropyl-β-D-thiogalactopyranoside for 2–3 h. The induced bacteria were resuspended in phosphate-buffered saline buffer (PBS) containing 1% Triton X-100 and lysed by sonication. Insoluble material was pelleted and supernatants were adjusted to 10% glycerol and stocked at −70°C. The bacterial lysate expressing GST fusion proteins was incubated with glutathione (GSH)-agarose beads (Sigma) at 4°C for 1 h and then extensively washed. GST-Vpr derivatives immobilized on GSH beads were incubated in a buffer containing 50 mM Tris-HCl pH 7.9, 0.2 mM EDTA, 0.5 mM PMSF, 2 mM DTT, 100 mM KCl, 0.3% NP-40 and 1 mg/ml BSA, in the presence of recombinant TFIIB (Santa Cruz Biotechnologies). After extensive washes, bound proteins were resolved by SDS-PAGE, electro-transferred to PVDF membranes (Amersham) and then processed for Western blotting using rabbit anti-TFIIB antibody (C18, Santa Cruz Biotechnologies) and horseradish peroxidase-linked swine anti-rabbit immunoglobulin (DAKO). Antibody binding was detected with ECL Western blotting detection reagents (Amersham). GST-TFIIB derivatives immobilized on GSH beads were incubated in a buffer containing 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.2% Tween-20 and BSA (200 μg/ml) in the presence of [^{35}S]Vpr. In vitro translated, radiolabelled Vpr was obtained as previously described [14]. Beads were extensively washed and bound proteins were resolved by SDS-PAGE and revealed by autoradiography.

2.4. V8 protease assay

This assay was performed as described [18]. 20 ng of recombinant TFIIB (Santa Cruz Biotechnologies) was incubated with increasing amounts (1, 2 or 4 μg) of GST derivatives immobilized on glutathione-agarose beads. The total protein concentration was maintained at 4 μg by the addition of GST-immobilized glutathione-agarose beads. Incubation was carried out for 1 h on ice in 40 mM HEPES, pH 7.5, 120 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT and 0.05% NP40, after which 10 ng of *Staphylococcus aureus* V8 protease (Sigma) was added and the samples were incubated at 30°C for an additional 15 min. Digestion was stopped by adding SDS-PAGE loading dye followed by incubation for 5 min at 100°C. Proteolytic fragments were resolved by 12% SDS-PAGE and visualized by immunoblotting using a rabbit polyclonal anti-TFIIB raised against a peptide corresponding to amino acids 299–316 mapping near the carboxy-terminus of human TFIIB (C18, Santa Cruz Biotechnologies) and horseradish peroxidase-linked swine anti-rabbit immunoglobulin (DAKO). Antibody binding was detected with ECL Western blotting detection reagents (Amersham).

3. Results and discussion

3.1. Vpr and TFIIB interact in cells

We have previously demonstrated that the HIV-1 Vpr protein functions as a transcriptional co-activator through interaction with the RNA polymerase II initiation factor TFIIB [14]. We investigated the biological relevance of these interactions by purifying protein-protein complexes present in cell extracts. GST or GST-Vpr fusion proteins were over-expressed in recombinant vaccinia virus-infected HeLa cells. The expression in cell extracts of ectopic Vpr and endogenous TFIIB was demonstrated by Western blotting (Fig. 1a). Cell extracts expressing GST or GST-Vpr were incubated with agarose-GSH beads. After extensive washes, bound proteins were eluted and separated by SDS-PAGE followed by Western blotting analysis with anti-TFIIB antibody. As shown in Fig. 1b, the TFIIB protein is present in the precipitated complexes containing GST-Vpr, and not in those containing GST, confirming that a specific interaction between TFIIB and Vpr

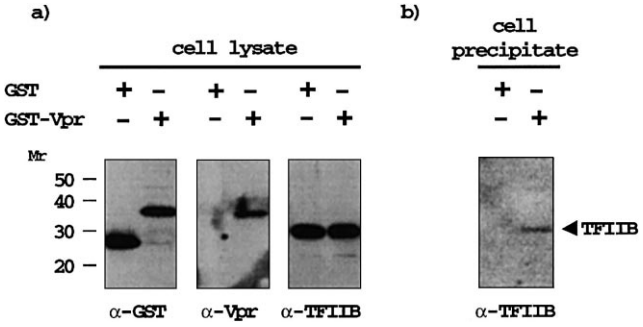

Fig. 1. Vpr and TFIIB co-precipitates in cells. Vaccinia virus-infected HeLa cells were transfected with expression plasmids encoding GST or GST-Vpr under the control of the T7 polymerase promoter. (a) Cells were lysed in lysis buffer and cell lysate was analyzed by Western blotting with anti-GST, anti-Vpr and anti-TFIIB antibody. (b) GST and GST-Vpr expressed in cell lysate were precipitated by addition of GSH-agarose beads. Beads were then extensively washed and bound proteins were resolved by SDS-PAGE and revealed with anti-TFIIB antibody. Mr: molecular mass markers (in kDa).

takes place in human cells. These data indicate that fusion of Vpr with GST does not affect its ability to associate with TFIIB. In agreement with these data, the Vpr-TFIIB interaction has recently been revealed by co-immunoprecipitation experiments and an increase of the Vpr-TFIIB interaction has been observed in the presence of dexamethasone [17].

3.2. The domain of Vpr involved in the Vpr-TFIIB interaction

Vpr contains in its N-terminal region an amphipatic α-helical domain spanning residues 17–34, which is important for incorporation of Vpr into viral particles [19–21]. A second α-helix containing a stretch of leucine/isoleucine residues acting as a leucine zipper and involved in the dimerization of Vpr has recently been reported by the analysis of the NMR-derived structure of the C-terminal domain of Vpr (residues 52–96) [22]. The C-terminal domain of Vpr contains several basic residues which are involved in the Vpr-mediated cell cycle arrest [19,23,24]. We investigated a series of Vpr-mutated proteins for their ability to bind to TFIIB. The *vpr* genes containing appropriate substitutions were inserted into a GST fusion expression vector and bacterially expressed as GST fusion proteins. In vitro protein-protein interactions were performed by incubation of similar amounts of GST-Vpr mutant fusion proteins with recombinant TFIIB. Bound proteins were resolved by SDS-PAGE and revealed by Western blotting with anti-TFIIB antibody. As shown in Fig. 2a, wild-type Vpr, E25K, A30F and V57L Vpr mutants bind to TFIIB as efficiently as wild-type Vpr. In contrast, the R80A Vpr mutant was clearly affected in its binding to TFIIB. These results indicate that a point mutation in the C-terminal basic domain of Vpr significantly impaired the Vpr-TFIIB interaction.

3.3. The domain of TFIIB involved in the Vpr-TFIIB interaction

Using a phage display assay, we have recently shown that a consensus WxxF motif is a target for Vpr [25]. Amino acid sequence analysis of TFIIB reveals that a WxxF motif is located in the N-terminus of the molecule (residues 52–55). We constructed a series of GST-TFIIB proteins mutated in the WxxF motif, either at the W and/or F residues (W52A, F55A

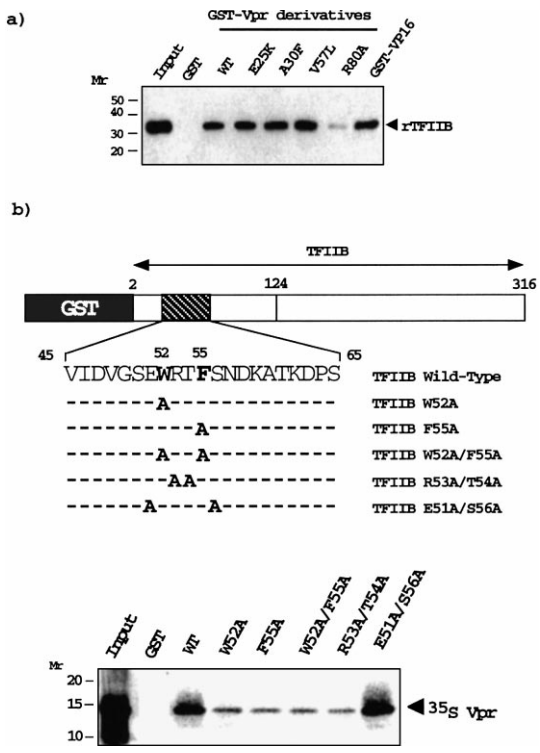

Fig. 2. Domains of Vpr and TFIIB involved in the Vpr-TFIIB interaction. (a) Equivalent amounts (2 μg) of GST and GST-Vpr mutated fusion proteins immobilized on GSH beads were incubated with 50 ng of recombinant TFIIB, bound proteins were resolved on SDS-PAGE and revealed by Western blotting with a rabbit polyclonal anti-TFIIB antibody. GST-VP16 protein was used as a positive control. Lane marked input represents 10 ng of rTFIIB. (b) Top panel: schematic diagram of the GST-TFIIB mutant proteins used to map the Vpr binding site in TFIIB. The region containing the WRTF motif in the N-terminal domain is represented as a hatched box and introduced point mutations are indicated. Bottom panel: equivalent amounts of GST and GST-TFIIB wild-type or mutated proteins were affinity-purified on GSH-agarose beads and incubated with the in vitro translated radiolabelled Vpr protein. Bound proteins were separated by SDS-PAGE and the gel was autoradiographed. The lane marked input contains 40% of [^{35}S]protein prior to binding experiments. Mr: molecular mass markers (in kDa).

and W52A/F55A) or at residues xx inside the motif (R53A/T54A) or at residues outside the motif (E51A/S56A) (Fig. 2b, top panel). GST, GST-TFIIB wild-type or mutated proteins were incubated with [^{35}S]Vpr and bound labelled proteins were resolved by SDS-PAGE and revealed by autoradiography. As shown in Fig. 2b, bottom panel, wild-type TFIIB binds Vpr, but the introduction of mutations in the WxxF motif of TFIIB dramatically decreased the Vpr binding, while mutations located outside the motif had no effect on the binding of Vpr.

Interestingly, it has been reported that native TFIIB is in a 'closed' conformation due to an intramolecular bridge between its N-terminus (residues 24–65) and the C-terminus (residues 202–297) [18]. Disruption of the TFIIB intramolecular bridge upon binding of the herpes virus VP16 transactivator induces a change in the conformation of TFIIB. This conformational change in TFIIB has been proposed to facilitate further assembly of TFIIF and RNA polymerase II allowing stimulation of transcription. Our data indicating that the position of the Vpr binding site in the N-terminus of TFIIB (residues 52–55) overlaps the domain involved in the intramolecular bridge (residues 24–65) suggest that Vpr may have the ability to induce a change of conformation in TFIIB.

3.4. Vpr induces a conformational change in TFIIB

Recombinant TFIIB was subjected to limited digestion with *S. aureus* V8 protease, in the presence of increasing amounts of GST-Vpr wild-type or GST-Vpr-mutated proteins immobilized on glutathione-agarose beads as previously described [18]. After incubation, proteolytic products were resolved by SDS-PAGE and visualized by immunoblotting with an antibody raised against the C-terminal part of TFIIB. As shown in Fig. 3a, incubation of TFIIB with protease and increasing amounts of GST-Vpr wild-type, but not GST, led to the induction of a single proteolytic cleavage product which was Vpr concentration-dependent (filled arrow), in addition to the initial proteolytic cleavage products (approximately 27–28 kDa and 17–18 kDa, open arrows). The difference in the TFIIB digestion pattern must reflect differences in the accessibility of V8 protease to proteolysis sites in the TFIIB-Vpr complexes. The slight variations of the intensity of the initial proteolytic cleavage products of TFIIB were Vpr concentration-independent and therefore not induced by Vpr. Moreover, we assume that it is likely the binding of Vpr to TFIIB which masked the 27–28 kDa product because this product remains unchanged in the presence of the R80A Vpr mutant which is impaired for binding to TFIIB (Fig. 3b). The Vpr-induced proteolytic cleavage product was not observed when TFIIB was incubated with 4 μg of GST-Vpr in the absence of protease (data not shown). The size of the Vpr-induced fragment indicates that the proteolytic cleavage site is located in the N-terminal domain of TFIIB. Under the same conditions, incubation of TFIIB in the presence of protease and increasing amounts (1, 2 and 4 μg) of GST-VP16, but not GST, led to the induction of a major TFIIB proteolytic cleavage product which was VP16 concentration-dependent, confirming previous data [18] indicating that VP16 has the ability to change the conformation of TFIIB (data not shown).

We next investigated in this proteolysis assay some Vpr-mutated proteins, previously defined for their ability to stimulate transcription [26]. As shown in Fig. 3b, E25K and V57L Vpr proteins were able to induce increasing amounts of the TFIIB proteolytic cleavage product (filled arrow). The Vpr R80A mutant which was impaired to bind to TFIIB was impaired to induce a TFIIB proteolytic cleavage product, indicating that the Vpr-induced proteolytic cleavage site was dependent on the interaction between Vpr and TFIIB. Although able to bind to TFIIB, increasing amounts of the Vpr A30F mutant did not induce increasing amounts of a proteolytic cleavage product, indicating that this mutant was unable to induce a conformational change in TFIIB. Altogether, these data indicate that E25K and V57L Vpr mutants, but not A30F and R80A Vpr mutants, are fully competent to bind to and to induce a conformational change in TFIIB.

A correlation between the induction of a conformational change in TFIIB and the stimulation of transcription has been reported [18]. E25K and V57L Vpr mutants have been reported to stimulate transcription of HIV-1 LTR as efficiently as wild-type Vpr, while A30F and R80A Vpr mutants were significantly impaired to stimulate transcription [26]. Interestingly, our results indicate a direct correlation between the ability of Vpr to induce a conformational change in TFIIB

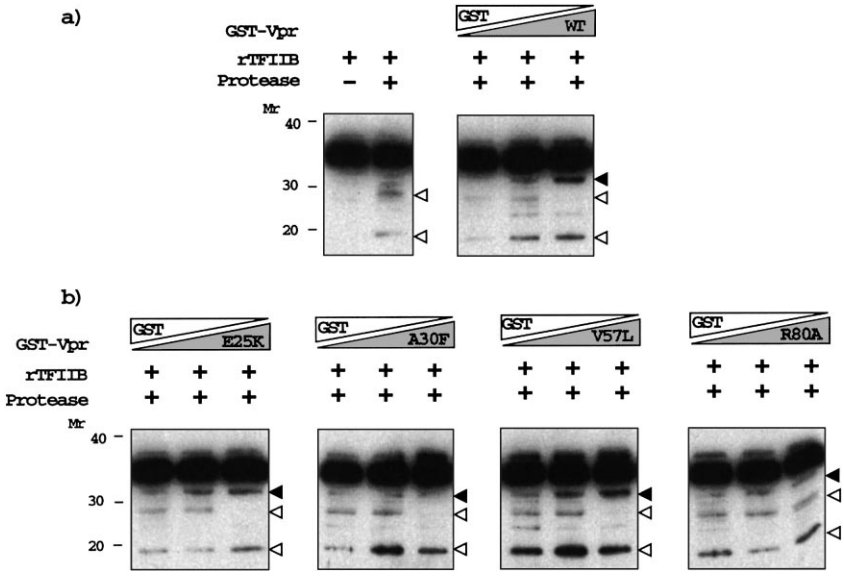

Fig. 3. HIV-1 Vpr induces a conformational change in TFIIB. Recombinant TFIIB (20 ng) was mixed with increasing amounts (1, 2 and 4 μg) of GST derivatives immobilized on glutathione-agarose beads and incubated with 10 ng of *S. aureus* V8 protease. The total protein concentration was held constant by addition of non-fused GST protein immobilized on glutathione-agarose beads. Proteolytic cleavage products were resolved by SDS-PAGE and revealed by Western blotting using a rabbit polyclonal antibody raised against the residues 299–316 of TFIIB. (a) Induction of the conformational change in TFIIB incubated with increasing amounts of GST-Vpr wild-type. The filled arrow indicates the Vpr-induced proteolytic cleavage product of TFIIB. Open arrows indicate the initial proteolytic cleavage products in TFIIB, induced by the V8 protease. (b) Induction of the conformational change in TFIIB, incubated with increasing amounts of GST-Vpr-mutated proteins. Mr: molecular mass markers (in kDa).

and its ability to stimulate transcription. Indeed, E25K and V57L Vpr mutants which were able to induce a TFIIB conformational change were able to transactivate. A30F and R80A Vpr mutants which were impaired to induce a TFIIB conformational change were significantly impaired to transactivate. These data provide a possible molecular mechanism by which Vpr mediated the HIV-1 LTR transcription through induction of a functional change in the conformation of TFIIB. The possibility that Vpr stimulates transcription through a distinct pathway cannot be ruled out. Indeed, it has been reported that the R80A mutation in Vpr, although affecting the Vpr-mediated transcription of HIV-1 LTR, has no effect on the Vpr-mediated transcription of the glucocorticoid responsive genes [17,27]. The opposite effects in the stimulation of transcription observed for this mutant seems to be dependent on the nature of the promoter, suggesting that Vpr may activate the transcriptional process by alternative pathways. The Vpr-mediated transcription of the glucocorticoid responsive genes in the presence of dexamethasone requires a direct interaction of Vpr with the promoter-bound glucocorticoid receptor. It is likely that the interaction of Vpr with the activator may be more important for stimulation of transcription than the interaction of Vpr with TFIIB. In agreement with this hypothesis, the R80A Vpr mutant which binds to the glucocorticoid receptor, and not to TFIIB, still retains its ability to transactivate glucocorticoid responsive genes. In contrast, the L64A Vpr mutant, which is impaired to bind to the glucocorticoid receptor, but still binds to TFIIB, is impaired for the glucocorticoid-activated transcription.

The contribution of the co-activator activity of Vpr remains to be defined in the context of HIV-1 replication. The viral genome-encoded transactivator Tat and several cellular transcription factors are required for efficient HIV-1 expression [28]. It is unknown how the viral genome initiates early transcription of the viral genes immediately after viral entry when Tat is at a suboptimal level. Vpr is a virion-associated protein and consistent with this localization, it could play a critical role in HIV-1 replication during early stages of HIV-1 infection. It is possible that Vpr affects qualitatively TFIIB assembly by inducing the conformational change in TFIIB, driving pre-initiation complex assembly forward. The weak Vpr-induced level of transcription could be sufficient to allow Tat expression and full stimulation of the HIV-1 LTR transcription. Whether the expression of Vpr affects also quantitatively TFIIB assembly by recruiting TFIIB into the pre-initiation complex will require further studies. In addition to its role in modulating the transcription of HIV-1 LTR, it is conceivable that the co-activator function of Vpr affects the transcription of several cellular promoters thus deregulating cellular gene expression in infected cells. Indeed, Vpr has been demonstrated to suppress some cytokines expression through its ability to indirectly alter the NF-KB activity [29]. Vpr has been reported to be a secreted protein [30,31] suggesting that it could deregulate cellular gene expression in uninfected cells contributing to the pathogenesis of AIDS.

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