Extent of incorporation of HIV-1 Vpr into the virus particles is flexible and can be modulated by expression level in cells

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Abstract To examine the factors that control the extent of incorporation of Vpr into the virus particles, we utilized an epitope-tagging approach with Flag (FL) as the epitope for quantitation. We generated expression plasmids containing Vpr-FL and Vpr E21,24P-FL and also HIV-1 proviral DNA containing Vpr-FL (NL-Vpr-FL). Immunoblot analysis using Flag antibodies revealed that virus particles derived from co-transfection of NL-Vpr-FL and Vpr-FL showed an enhanced level of Vpr-FL in comparison to NL-Vpr-FL derived virus. These results suggest that the amount of incorporation of Vpr into the virus particles is flexible and may be modulated by its expression level in cells.

Key words: HIV-1; Non-structural viral protein; Virion specific; Trans-expression of Vpr

1. Introduction

The biochemical analysis of virus particles encoded by the HIV-1 genome revealed the presence of both structural proteins, common to all retroviruses, and non-structural proteins of viral origin [10,15,28]. The structural proteins in the virus particles are Gag, Gag-pol and the Env [8,23]. The Env protein reaches the cell surface through the secretory pathway and both Gag and Gag-pol arrive at the cell membrane with the help of a myristylation signal through an unknown mechanism [23]. The non-structural proteins present in the virus particles include Vif, Vpr, and Nef [2–4,6,9,10,18,24, 28,39,40,53]. Of the non-structural proteins, only Nef possesses a myristylation signal similar to Gag and Gag-pol [28]. Interestingly, the non-structural proteins have been shown to be present in different amounts in the virions raising questions about the mode of incorporation of these proteins [16,19]. In this regard, recent studies showed that Vif and Nef are incorporated into the virus particles by a non-specific mechanism [3,4,19,44,49]. A recent study reported that Vif is not present in highly purified virions [12]. On the other hand, Vpr incorporation is mediated by a specific mechanism involving Vpr and Gag [5,26,27,31,40].

In an effort to study the underlying factors that contribute to the incorporation of non-structural proteins into the virus particles, we have been conducting studies on Vpr for the past several years. Work from our laboratory and others showed the importance of the putative helical domains in Vpr for its incorporation into virus particles [13,14,32–37,45,52]. With respect to the specificity of Vpr for HIV-1 virions, it was shown that Vpr requires the p6 region of Gag, as the deletion of this domain completely eliminated its incorporation [5,25,26,31]. Further, the addition of this motif to Moloney murine leukemia virus Gag enabled the incorporation of Vpr into the virus particles [26]. Despite a wealth of date available on Vpr, the information regarding the number of molecules of Vpr within the virus particles is not clear. The studies reported in the literature indicate contradictory data regarding the number ranging from 250–2500 molecules [9,25].

Based on the specific incorporation feature of Vpr for HIV-1 virions, it has been considered as a potential carrier of proteins/peptides of interest into the virus particles [17,25,38,43,48,50,51]. In this regard, it was shown that the generation of a chimeric Vpr containing Vpr and HIV-1 protease cleavage recognition sequences, resulted in the inhibition of virus replication [43]. The effectiveness of this as a therapeutic approach depends on the incorporation of chimeric Vpr into the virus particles in the presence of wild-type Vpr. Hence, a central question regarding this approach is whether incorporation of Vpr with respect to the number of molecules is rigid or the extent of incorporation can be modulated by the expression level of Vpr in cells. To explore this, we have employed HIV-1 proviral DNA containing Vpr-FL and an expression plasmid of the same in which Vpr-FL coding sequences are under the control of the immediate early promoter of human cytomegalovirus. The analysis of virus particles indicated that the extent of incorporation of Vpr can be altered with the expression level of Vpr in cells.

2. Materials and methods

2.1. Construction of Vpr-FL expression plasmid and generation of HIV-1 proviral DNA containing Vpr-FL

Vpr-FL and Vpr E21,24P-FL expression plasmids were generated by using PCR methodologies [33,42,43]. The sequences corresponding to the Flag epitope (DYKDDDDK) [7] were incorporated in the 3′ primer. DNA fragments were amplified using the proviral clone NL4-3 as the template and cloned into the pCDNA$_3$ vector between *Hin*dIII and *Xho*I restriction enzyme cleavage sites. The coding sequences in the vector are flanked by the human cytomegalovirus (CMV) immediate early promoter and T7 promoter sequences upstream and bovine growth hormone poly(A) signal sequences downstream. Vpr-

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FL coding sequences were amplified with the primer pair designated HKVpr(+) forward, 5′-TCTAGAAGCTTGCCGCCACCATGGAACAAGCCCCAGAAGAC-3′ and Vpr-FL(−) reverse, 5′-CCCCCCTCGAGCTACTTGTCATCGTCGTCCTTGTAGTCGGATCTACTGGCTCCATT-3′. The primers used for point mutations in Vpr E21,24P-FL were (the nucleotides differing from the parent plasmid are shown boldface) forward, 5′-**CC**GCTTTTA**CC**GGAGCTTAAGAATGAAGCTGTGAGA-3′ and reverse, 5′-AAGCTCC**GG**TAAAGC**GG**TAGTGTCCAGTCATTGTA-3′. All recombinant plasmids were verified by restriction enzyme cleavage and DNA sequence analysis.

Vpr-FL containing pCDNA$_3$ vector was cleaved with *Eco*RI and *Xho*I to generate the fragment for insertion into proviral DNA (NL4-3) cleaved at *Eco*RI and *Sal*I as described [43]. The proviral DNA designated NLΔVpr lacks Vpr expression due to a frameshift mutation (close to *Eco*RI recognition site) in the coding sequences.

2.2. In vitro transcription/translation and radioimmunoprecipitation analysis of proteins

The coupled T7 transcription/translation system (Promega, Madison, WI, USA) was used for characterizing the expression of the recombinant clones. Incubation conditions were followed according to the manufacturer's instructions. Radioimmunoprecipitation analysis (RIPA) of in vitro translated proteins was carried out using polyclonal sera to Vpr and the Flag epitope [43].

2.3. Generation of virus particles directed by HIV-1 proviral DNA

HIV-1 proviral DNA (wild-type and modified in Vpr coding sequences) (5 μg) was transfected into RD cells as described [43]. Virus particles released into the culture medium were harvested 72–120 h post-transfection and quantitated by p24 antigen assay using an ELISA (Organon Teknika, Durham, NC, USA).

2.4. Immunoblot analysis

Virus containing culture supernatants were pre-cleared for 10 min at 10 000 rpm and subsequently spun at 40 000 rpm for 3 h using sucrose density gradient centrifugation. Virus pellets were lysed with phosphate buffered saline (PBS) containing 0.05% Triton X-100. Samples, normalized on the basis of p24 antigen values, were run on 10%/16% SDS–PAGE gel followed by transfer to a nitrocellulose membrane [42]. After each antibody incubation, blot was washed several times with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) (Bio-Rad). The CDP-Star™ (Promega) was used as the chemiluminescent substrate in a non-radioactive detection system.

2.5. Quantification by densitometry

The intensity of the bands obtained through immunoblot analysis was used for calculating the extent of incorporation of Vpr. Blots were scanned and densitometric analysis was performed using the Image Quant program (Molecular Dynamics) as described [42].

3. Results

3.1. Construction and expression of chimeric Vpr-FL

The Vpr coding sequences were cloned into a plasmid vector which contains the immediate early promoter derived from human cytomegalovirus. To enable the detection and quantitation of Vpr, an epitope tag approach was utilized. Flag epitope (DYKDDDDK), for which polyclonal and monoclonal antibodies are commercially available, was added to the C-terminus of Vpr (Fig. 1), using PCR, to generate Vpr-FL. We have also generated a mutant Vpr in which the glutamic acid residues located at positions 21 and 24 of Vpr were replaced with proline residues. The mutant Vpr containing proline residues showed a drastic reduction in its ability to be incorporated into the virus particles [34]. Hence, using mutant Vpr would serve as a control with respect to specific incorporation of Vpr. The backbone plasmid vector, pCDNA$_3$, was used as a negative control. To examine the protein directed by the Vpr-FL and Vpr E21,24P-FL plasmids, an in vitro transcription/translation system was used. The protein synthesized

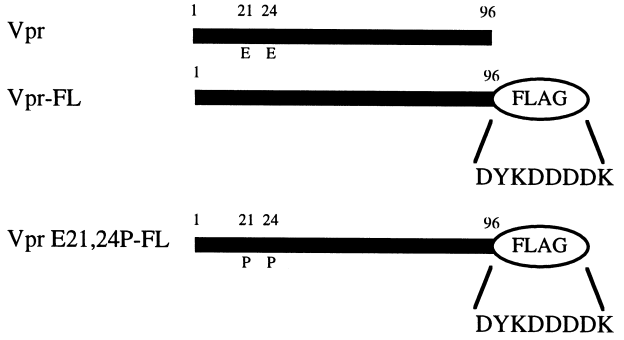

Fig. 1. Schematic representation of Vpr and Vpr-FL and Vpr E21,24P-FL. The sequences corresponding to the Flag epitope are added to the 3′ end of Vpr.

in vitro was immunoprecipitated using antiserum against Vpr (Fig. 2A) and the Flag epitope (Fig. 2B). The analysis of the proteins on SDS–PAGE showed that Flag antibodies detected a protein of 14 kDa in size which was also reactive to Vpr anti-serum. As expected, a band corresponding to the protein in the same molecular weight range was not detected with the backbone pCDNA$_3$ vector in the reaction.

3.2. Assessment of the specificity of incorporation of Vpr into the virus particles

The specific incorporation of Vpr directed by HIV-1 is a prerequisite for determining the amount of Vpr present within the virus particles. We considered the possibility that non-specific associations of Vpr with contaminating vesicles in the virus preparation may lead to a wrong estimation. To rule this out, we utilized wild-type and mutant Vpr in co-transfection experiments with HIV-1 proviral DNA lacking Vpr expression (NLΔVpr). The immunoblot analysis of virus particles showed that virion incorporation was observed for

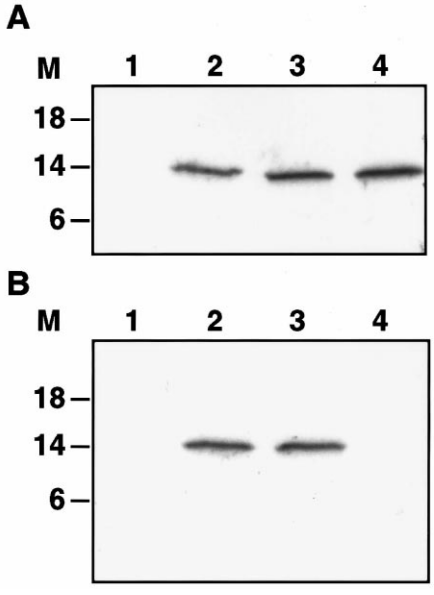

Fig. 2. Radioimmunoprecipitation analysis of the protein generated through in vitro transcription/translation system using polyclonal sera to (A) Vpr, and (B) Flag epitope. M, molecular weight markers (kDa); lane 1, pCDNA$_3$; lane 2, Vpr-FL; lane 3, Vpr E21,24P-FL; lane 4, Vpr.

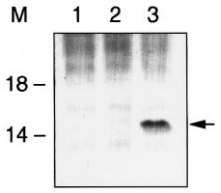

Fig. 3. Immunoblot analysis of virus particles derived from transfected cells using antibodies against Flag epitope. Equal amounts of virus lysate, normalized on the basis of p24 antigen values, were separated on SDS–PAGE. M, molecular weight markers (kDa); lane 1, $pCDNA_3$; lane 2, NLΔVpr+Vpr E21,24P-FL; lane 3, NLΔVpr+Vpr-FL. Arrow indicates Vpr-FL.

wild-type Vpr and that mutant Vpr failed to incorporate into the virus particles under the conditions used (Fig. 3). These experiments clearly indicate that there is not any non-specific association of Vpr-FL with the virus particles, as reported for wild-type Vpr [34,52].

3.3. Generation of HIV-1 proviral DNA containing Vpr-FL

We have adapted a strategy which was successfully used in an earlier study by our group [43], to introduce chimeric Vpr sequences into HIV-1 proviral genome. The 3′ end of the Vpr coding region contains a unique cleavage site for the restriction enzymes *Eco*RI and *Sal*I, which are located at residues 62 and 76, respectively. DNA sequence analysis reveals an overlap of Vpr and Tat involving only 24 nucleotides at the 3′ end of Vpr. Considering this, an insertion of *Eco*RI-*Xho*I fragment from the Vpr-FL expression plasmid into the proviral DNA cleaved with *Eco*RI and *Sal*I (NL-Vpr-FL) may not create an undesirable effect. In this scheme, even though 20 amino acid residues at the C-terminus of Vpr are duplicated, the second copy will not be translated because of a termination codon following the first copy.

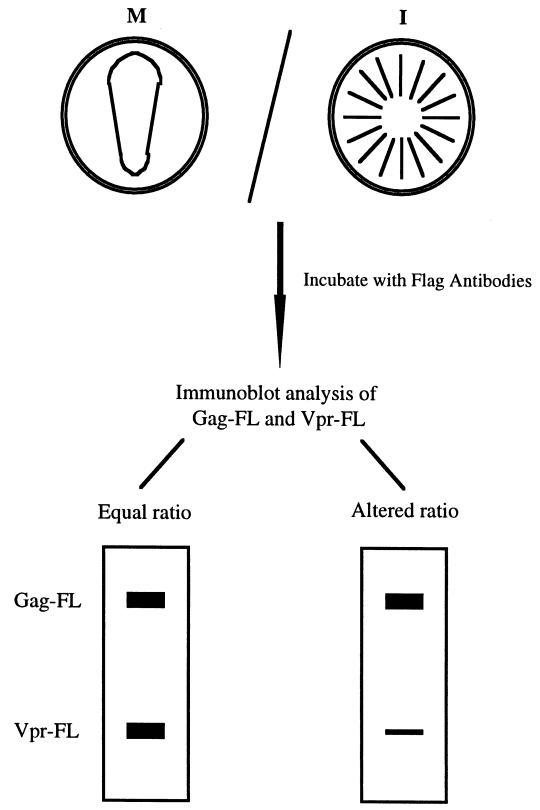

Fig. 4. Hypothetical scenarios for the extent of incorporation of Vpr into the virus particles.

3.4. Analysis of epitope-tagged Vpr in the virus particles

To monitor the extent of incorporation of Vpr-FL into the virus particles in relation to the amount of Vpr-FL present in cells, we have considered a strategy involving co-transfection of NL-Vpr-FL proviral DNA with Vpr-FL expression plasmid. The analysis of virus particles derived from the co-transfection of DNA into cells may reveal one of two possible scenarios. It is likely that the amount of Vpr is low in cells when Vpr is expressed in the context of HIV-1 proviral DNA. The low amount of Vpr-FL in the virus particles may be reflective of the level within the cells (Fig. 4). On the other hand, the expression of Vpr via the CMV immediate early promoter could lead to an increased level of Vpr inside the cells. The increased availability of Vpr may result in an enhanced level of Vpr-FL incorporation into the virus particles (Fig. 4). Alternatively, the incorporation remains constant, showing no correlation with the level of Vpr inside the cells. To experimentally verify this, the proviral DNAs NL4-3 and NL-Vpr-FL were transfected into RD cells and the virus particles were collected between 72–120 h after transfection. The

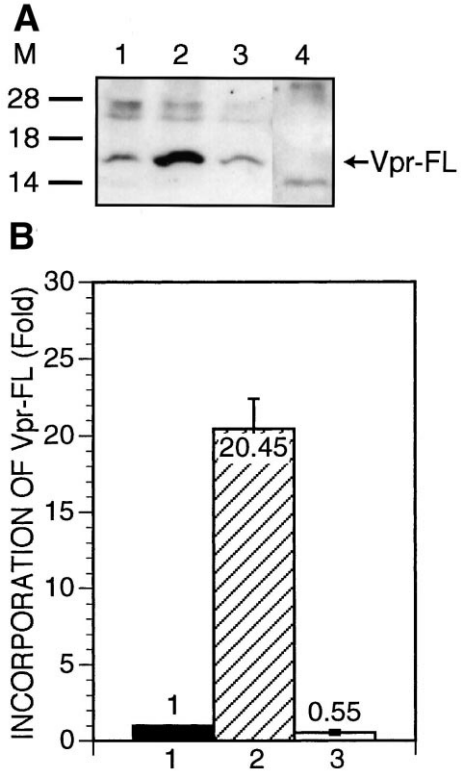

Fig. 5. A: Immunoblot analysis of virus particles derived from the DNA transfected cells using antibodies against Flag epitope. The virus particles were harvested 120 h after transfection and virus lysates normalized on the basis of p24 antigen values were loaded on the gel. M, molecular weight markers (kDa); lane 1, NL-Vpr-FL; lane 2, NL-Vpr-FL+Vpr-FL; lane 3, NL-Vpr-FL+Vpr; lane 4, NL4-3. B: Densitometric analysis of Vpr-FL in the virus particles. the data represent the average of two independent experiments as described.

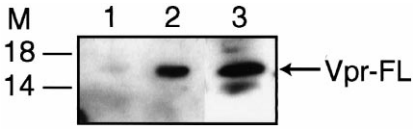

Fig. 6. Immunoblot analysis of virus particles using antibodies against Flag epitope. M, molecular weight markers (kDa); lane 1, NLΔVpr; lane 2, NLΔVpr+Vpr-FL; lane 3, Vpr-FL (positive control). An equal amount of viral lysate based on p24 antigen was loaded.

viral lysate, upon p24 antigen assay, was subjected to immunoblot analysis using Flag antibodies. The results showed a band corresponding to Vpr-FL in the virus particles derived from NL-Vpr-FL and not from cells transfected with NL4-3. The incorporation level of Vpr-FL, as shown in Fig. 5A, is low when expressed in the context of proviral DNA. However, the virus particles derived from co-transfection of NL-Vpr-FL and the Vpr-FL expression plasmid showed an intense Vpr-FL band in comparison to NL-Vpr-FL suggesting that the amount of Vpr-FL incorporated into the virus particles is flexible.

To address the specificity and competition between Vpr molecules, co-transfection of NL-Vpr-FL with the Vpr wild-type expression plasmid (lacking the Flag epitope) was carried out. It was reasoned that the incorporation of Vpr without the Flag epitope will reduce the signal observed for Vpr-FL incorporated into the virus particles. The immunoblot analysis of virus particles revealed a less intense band in support of this (Fig. 5A, lane 3).

The densitometric analysis of the immunoblot signals, upon normalization based on p24 antigen values, showed a 20-fold increase in the incorporation of Vpr-FL in co-transfection experiments in comparison to NL-Vpr-FL alone (Fig. 5B). Such an analysis involving virus particles derived from NL-Vpr-FL and a Vpr expression plasmid showed a signal which was 45% less than that of NL-Vpr-FL.

These results support the hypothesis that at least one of the limiting factors in the level of incorporation of Vpr-FL is the amount of Vpr-FL present within cells. This conclusion was also supported by the analysis of virus particles derived from co-transfection of NLΔVpr which lacks the ability to synthesize Vpr, and Vpr-FL expression plasmids. The expression of Vpr-FL, in *trans*, resulted in an enhanced incorporation of Vpr-FL within the virus particles (Fig. 6), in comparison to the virus from NL-Vpr-FL.

4. Discussion

The studies described here provide evidence that one of the contributing factors underlying the incorporation of non-structural proteins such as Vpr into the virus particles is the level of expression of the protein within the cell. The increased level of expression of Vpr correlated with its enhanced incorporation into the virus particles. Vpr, one of the three auxiliary proteins present inside the virus particles, is regulated by Rev [10,28]. It is likely that the low amount of Vpr noted within the virus particles derived from NL-Vpr-FL is a consequence of the low level of Vpr present within cells. A related issue that needs to be addressed is whether the extent of incorporation of a non-structural protein is fixed or flexible in regards to the amount of structural protein Gag (Fig. 4). Co-transfection of HIV-1 proviral DNA containing Vpr-FL with the Vpr-FL expression plasmid was carried out to derive the virus particles for the analysis. An enhanced incorporation of Vpr-FL was detected by immunoblot analysis of virus particles and such an increase was not noted when $pCDNA_3$ DNA was used for co-transfection. The increased Vpr-FL incorporation was dependent upon the concentration of the expression plasmid used for co-transfection (data not shown).

Though incorporation of Vpr into the virus particles has been noted by several investigators, the underlying mechanism involved remains unclear [9,10,14,15,30,31,34,40,47,53]. In this regard, clearly the amount of Vpr present in relation to Gag in the virus particles may provide clues as to a possible mechanism(s). Studies reported in the literature suggested that direct interaction between Vpr and Gag may be responsible for the incorporation of Vpr into the virus particles. Lavellee et al. [27] provided evidence in support of the interaction of Gag and Vpr in cells. Recently, interactions between Vpr and p17 was noted in a yeast two-hybrid system [41]. Similarly, Vpr interactions with NCp7 was also reported [11,46]. Though these studies showed an interaction of Vpr with processed components of the Gag precursor Pr55, it remains to be demonstrated whether such interactions exist involving the precursor protein. This is important in light of the fact that both NMR and crystallographic studies of matrix and capsid of HIV-1 Gag suggest conformational changes upon cleavage of the precursor protein [19–22].

With respect to the incorporation of non-structural proteins, the number of molecules present in the virion has been estimated for Vif (7–100 molecules) [2,4,19,29] and Nef (5–20 molecules) [49]. The information for Vpr is less clear. The epitope-tagging approach considered here has revealed the following new information: (i) the number of molecules of Vpr incorporated into the virus particles is not rigid, (ii) incorporation of Vpr can be modulated by the level of Vpr in cells, (iii) extent of incorporation of Vpr into the virus particles is directly proportional to the level of expression in cells, (iv) co-transfection of NLΔVpr and Vpr-FL expression plasmids also showed an enhanced incorporation of Vpr-FL into the virus particles in comparison to the expression of Vpr-FL in the context of proviral DNA, and (v) the inclusion of a Vpr mutant (Vpr E21,24P-FL), which exhibits a negative virion incorporation phenotype, in co-transfection experiments using NLΔVpr indicated that increased Vpr-FL was not due to non-specific association with the virus particles.

In addition to providing information regarding the incorporation level of Vpr when expressed in *cis* or *trans*, the results generated here have a bearing on the stoichiometry of the proteins in the virus particles. Analysis of HIV-1 particles has led to the suggestion that there are 2750 molecules of Gag present in a virion [1]. Gag-pol protein generated by a ribosomal frameshifting mechanism was estimated to be 10–20-fold less than that of Gag in the virus particles [8]. While the amount of structural proteins (both Gag and Gag-pol) present in the virus particles is rigid with respect to the number, the results obtained for Vpr in this study suggest a flexible mode regarding the incorporation of non-structural proteins into the virus particles. Recently, a similar scenario was reported for Vif [44]. However, the difference between Vif and Vpr is that the former utilizes a non-specific mechanism for its incorporation into the virus particles. It is unclear at this time whether the number of molecules of Nef is rigid or flexible.

This information may help to draw a general conclusion that the rules for the incorporation of non-structural proteins into the virus particles are different from the structural proteins.

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