Human Immunodeficiency Virus Vpx Is Required for the Early Phase of Replication in Peripheral Blood Mononuclear Cells

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Abstract: Functional importance of Vpx protein of human immunodeficiency virus type 2 was evaluated in various types of cells. In 8 lymphocytic or monocytic cell lines tested, vpx mutant virus grew as well as wild-type virus. Only in primary peripheral blood mononuclear cell cultures, severely retarded growth of mutant virus was observed. No replication of vpx-minus virus was detected in primary macrophage cells. A highly sensitive single-round replication assay system was used to determine the defective replication phase in primary mononuclear cells of vpx mutant virus. In all cell lines examined, vpx mutant displayed no abnormality. In contrast, the vpx mutant was demonstrated to be defective at an early stage of the infection cycle in primary cell cultures. No evidence of a replication-defect at a late phase in primary cells of the vpx mutant was obtained by a transfection-coculture method. These results indicate that the virion-associated Vpx protein is essential for early viral replication process in natural target cells such as primary macrophages.

Key words: HIV-2, Vpx

The genomes of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) are complex and contain several extra genes which modulate viral replication in vitro (5, 7) and in vivo (17, 19). HIV-2 carries one of these genes designated vpx which is specific for viruses of the HIV-2 and SIVAGM (virus from African green monkeys) groups (6). The vpx gene encodes a 12-16 kDa protein which is a structural component of the virus particle, and the Vpx protein is present in near equimolar amounts in virion compared to the major structural protein gag p24 (14). Previous mutational analyses of HIV/SIV have demonstrated that the Vpx protein is not required for infection and growth in established cell lines of lymphocytic or monocytic lineage (12, 15, 16, 29, 34). The importance of the HIV-2 Vpx protein for viral replication is shown in human peripheral blood mononuclear cells (PBMCs) (2, 12, 16). In normal PBMCs, vpx-deficient viruses replicated at substantially lower titers. This defect was reported to be localized to early events in the viral life cycle since vpx-defective virus displayed several-fold reduction in viral DNA synthesis monitored by polymerase chain reaction (PCR) amplification in acutely infected cells (16).

To clarify the requirement for the Vpx protein in HIV-2 replication and to examine its function, we performed a detailed functional analysis in this report. Using isogenic wild-type and mutant viruses, we determined their relative replication potential in established lymphoid cell lines not examined in previous studies and in primary human blood cells. We also utilized a newly established single-round replication assay system (24, 25) to quantify differences in early and late HIV-2 replication phases.

Materials and Methods

Cells. A human colon carcinoma cell line SW480 (1) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated

Abbreviations: CAT, chloramphenicol acetyltransferase; CPE, cytopathic effect; FCS, fetal calf serum; HIV, human immunodeficiency virus; HIV-2, HIV type 2; M4-8, Molt4 clone 8; nt, nucleotide; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; RT, reverse transcriptase; SIV, simian immunodeficiency virus; SIVAGM, SIV isolated from African green monkey; wt, wild-type.
fetal calf serum (FCS). CD4⁺ human lymphoid cell lines listed in Table 1 were maintained in RPMI-1640 medium containing 10% FCS. Human PBMCs were prepared from heparinized whole blood, stimulated with 10 μg of concanavalin A per ml for 24 hr, and maintained in RPMI-1640 medium containing 10% FCS and human interleukin-2 as described previously (22). Primary human macrophage cells were prepared from PBMCs as described previously (10).

Transfection. SW480 cells and PBMCs were transfected by the calcium phosphate coprecipitation (1) and modified DEAE-dextran (31) techniques, respectively.

Infection. Culture supernatants of virus-producing SW480 cells were filtered (0.45-μm pore size), and appropriate volumes were added to target cells as described previously (9).

RT assays. Virion-associated reverse transcriptase (RT) activity was measured as described previously (33). For quantitation, spots on DE81 paper were cut out, and RT activity was determined by scintillation counting.

CAT assays. The chloramphenicol acetyltransferase (CAT) assay has been previously described (11). CAT levels were assayed in equivalent amounts of cell lysates from infected cells.

DNA constructs. All molecular clones were constructed by standard recombinant DNA techniques. An infectious proviral clone of HIV-2, designated pGHI23, and its vpx mutant pGH-St have been previously described (29). Another infectious clone of HIV-2 with a wider host range, pGL-AN, and its vpx mutant, pGL-St, were constructed as shown in Fig. 1a. For single-round replication assays (Fig. 5), various proviral CAT constructs, designated pGHnCAT, pGHnCAT-St, pGHnCAT-Ns, pGLnCAT, pGLnCAT-St, and pGLnCAT-Ns (Fig. 1), which are structurally similar to those for analysis of HIV-1 (24, 25), were generated.

Results

Growth Characteristics of vpx Mutant Viruses in Various Types of Cells

To examine the growth potential of vpx-defective mutant virus in a wide variety of cells, two full-length clones were used as parental wild-type (wt) infectious molecular clones. As shown in Fig. 1a, a newly constructed wt clone pGL-AN contained env sequence derived from a molecular clone (unpublished) of HIV-2ROD (3, 13), which has a wide host range. Its vpx mutant, designated pGL-St, was made to compare its growth property to that of the wt clone (Fig. 1a). Four molecular clones in Fig. 1a were transfected into SW480 cells (1), and 2 days later, culture supernatants were harvested for input viruses of infection experiments. Target cells for infection are listed in Table 1; these included several cell types not tested in previous reports on functional analyses of HIV-2 vpx mutant viruses.

Figure 2 shows growth kinetics of the 4 viruses in several lymphocytic cell lines. In H9, Molt4 clone 8

\[ \text{Fig. 2. Growth of vpx-defective viruses.} \]

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HIV-2 Vpx IN PBMC

Fig. 2. Growth kinetics of various viruses in lymphocytic cell lines. To prepare cell-free virus samples, SW480 cells were transfected with 20 μg of proviral DNA clones (Fig. 1a), and 48 hr later culture supernatants were collected and filtered. Cells (10^6) were infected with 5 x 10^5 RT units of cell-free virus, and RT production was monitored at the designated intervals. Cell lines and viruses used are indicated.

Fig. 3. Growth kinetics of various viruses in monocytic cell lines. Methods for infection were the same as described in the legend to Fig. 2. Cell lines used are indicated. Symbols: closed circles, GH123; open circles, GH-St; closed triangles, GL-AN; open triangles, GL-St; open squares, mock-infection.

Fig. 4. Growth kinetics of various viruses in primary blood cell cultures. PBMCs (3 individuals) and macrophage cultures (one individual) were prepared by the methods of Ohta et al [22] and Gartner et al [10], respectively. Macrophage cultures were trypsin-resistant, almost 100% positive for nonspecific esterase (Sigma), and 75% MAC-1-positive (Dainippon Seiyaku) when used for infection experiments. Cells (10^6 of PBMCs; confluent adherent macrophage cells in one well of a 24-well plate [Corning]) were infected with 5 x 10^5 RT units of cell-free virus samples as described in the legend to Fig. 2, and monitored for RT production at the designated intervals.

(M4-8), and Molt3 cells, especially in M4-8 cells, the growth rate and cytopathic effect (CPE)-causing ability of 2 wt viruses were somewhat different. However, in none of the 5 cell lines in Fig. 2 was a
significant growth difference of wt and its corresponding vpx mutant viruses noticed. In contrast to the lymphocytic cell lines, all three monocytic cell lines used in this study did not support productive infection of one of the wt viruses (GH123) (Fig. 3). Another wt virus, GL-AN, grew fairly well in all cell lines, but again no significant difference of growth rate between the wt and mutant viruses was observed (Fig. 3). Finally, human primary blood cell cultures were tested for their susceptibility to infection with the four viruses (Fig. 4). PBMCs were prepared from three individuals and used for infection experiments. Primary macrophage cultures were also prepared from one of the individuals (Fig. 4). Consistent with previous reports (2, 12, 16), vpx mutant viruses exhibited growth kinetics significantly retarded relative to that of wt viruses in PBMCs. In primary macrophage cultures, replication of vpx mutant viruses was not observed during the observation period (Fig. 4).

In total, the vpx mutants showed their replication defect only in primary blood cell cultures.

Determination of Defective Replication Phase of vpx Mutant Viruses

To determine the infection stage in PBMC when the Vpx protein is required, we have developed a system, designated a single-round replication assay, which is similar to that previously described by us for HIV-1 study (24, 25). As shown in Fig. 5, this...

**Fig. 5.** Methods used for monitoring defective replication phase of the vpx mutant (single-round replication assay).

For quantitative analysis of early viral replication phase, virions recovered from SW480 cells cotransfected with replication-defective rev-deficient various nCAT constructs (Fig. 1b) and Rev expression vector prev2 (26) are used. These virions are able to undergo, at most, the early phase of replication cycle (from adsorption to integration) in CD4-positive target cells for infection (24, 25). Early phase of viral replication can be monitored by the highly sensitive CAT assay (11). For analysis of late viral replication phase (from transcription to virion release) in cells, which are only poorly transfectable like PBMCs, progeny virion production in the coculture (transfected cells plus highly sensitive cells such as M4-8) as monitored by the very sensitive RT assay (33) is indicative of a normal proceeding of the late replication phase.

**Fig. 6.** Sensitivity of single-round infection assays. Cells (2 X 10^6) indicated were infected with various RT units of virions (2 X 10^5 [top], 5 X 10^5, 1 X 10^6, 2 X 10^6 [bottom]) derived from pGHnCAT constructs (Fig. 1b), and 3 days later, CAT activity in cells were determined. Reaction time for CAT assay was 2 hr (left) and 6 hr (right).
system utilized replication-defective \textit{rev}-minus proviral clones carrying \textit{CAT} gene in the \textit{nef} gene (Fig. 1b) for quantitative analysis of early phase infection process (from adsorption to integration) (24, 25). For quantitative estimation of late infection process (from transcription to virion formation and release) in this system, PBMCs were transfected with regular proviral clones (Fig. 1a), and cocultivated with highly virus-sensitive M4-8 cells since PBMCs were very poorly transfected with DNAs.

Figure 6 shows typical examples of quantitative analysis of the early infection process monitored by \textit{CAT} assays using \textit{env}-deficient proviral \textit{CAT} clones (Fig. 1b) as negative controls. In highly sensitive cell lines for virus infection (GH123 and GH-St) such as M4-8 and M8166 (Figs. 2 and 3), \textit{CAT} activity was readily detected, and was input-dose dependent. A relatively low level of \textit{CAT} activity was expressed in M8166 cells, but they produced more viruses than M4-8 cells following infection (Fig. 2). This result probably represented an efficient late infection process in M8166 cells. In Molt3 and U937 cell lines with low or no sensitivity to virus infection (GH123 and GH-St viruses in Figs. 2 and 3, respectively), \textit{CAT} activity was barely detectable.

A wide variety of cell types were acutely infected with various viruses derived from pGHnCAT and pGLnCAT constructs, and 3 days later, \textit{CAT} activity in cells were determined (Fig. 7). Consistent with the results of infection experiments in Figs. 2 and 3, no significant difference in \textit{CAT} activities directed by wt and \textit{vpx} mutant viruses were noted in all seven cell lines examined. These results were confirmed in repeated experiments. When three PBMC preparations from two individuals were used for infection targets of GHnCAT viruses, lower \textit{CAT} activity was directed by the \textit{vpx} mutant than that by the wt virus (Fig. 7). The relatively low \textit{CAT} activity induced by the \textit{vpx} mutant was reproducibly observed in PBMCs from two individuals, and estimated to be approximately 40% of that by the wt virus. Primary macrophage cultures were not analyzed because the two wt viruses grew relatively poorly in the cells (Fig. 4).

Results obtained by transfection/coculture experiments designed for analysis of late infection phase (Fig. 5) are shown in Fig. 8. Two PBMC preparations prepared from two individuals were transfected with molecular clones pGH123 or pGH-St (Fig. 1), and on the next day, cells were cocultured with

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Analysis of early viral replication phase by single-round infection assays. Cells (2 \times 10^6) were infected with 5 \times 10^5 RT units of cell-free virions derived from various nCAT constructs (Fig. 1b), and 3 days later, \textit{CAT} activity in cells indicated was determined. \textit{CAT} activity directed by mutant clones relative to that by wt clones are shown. Reaction time for \textit{CAT} assay was adjusted to obtain linearity.}
\end{figure}
M4-8 cells. Virus production in the cocultures as judged by RT activity was monitored for 2 to 3 weeks. In both cocultures, the vpx mutant virus grew exactly like the wt virus (Fig. 8). PBMCs, which had been killed by UV before transfection, gave no infectious virus. In addition, PBMCs transfected with an env-deficient clone designated pGH-Ns (29), generated no infectious virus.

Discussion

The results presented in this study demonstrate that the Vpx protein of HIV-2 is required, in certain cell type(s) such as macrophages, for the early viral replication phase (from virus entry to integration).

The vpx-deficient viruses replicated normally (Figs. 2 and 3) in various established cell lines (Table 1). Together with the data published previously (12, 15, 16, 29, 34), a total of 10 established lymphoid cell lines have been checked for their supportability of the growth of vpx mutant viruses, and no significant difference in the replication characteristics between wt and mutant viruses has been observed, at least in acutely infected cells. Impaired growth of the vpx mutant viruses was noticed only in PBMCs and primary macrophage cultures (Fig. 4). In particular, no virus replication was detected in the macrophage cells (Fig. 4).

By a newly constructed single-round replication assay system (Fig. 5), the defective step in virus replication cycle in PBMCs of the vpx mutants was determined. Simply by monitoring CAT activity, the early infection process was quantitatively estimated without a noise originating from multiple rounds of viral replication (Fig. 6). The early viral replication phase in a wide variety of cell types, including PBMCs, following acute infection was evaluated by this assay system (Fig. 7). Consistent with the data on viral replication kinetics, no significant difference in CAT activity in established cell lines infected with wt or mutant viruses was observed. In contrast, in PBMCs a lower level of marker CAT was produced by the vpx mutant relative to that by the wt virus (Fig. 7), indicating that the HIV-2 Vpx is required for the early replication stage. These results are in good agreement with

![Fig. 8. Kinetics of RT production in transfected PBMC cocultivated with M4-8 cells. PBMCs (10^6) obtained from 2 individuals were transfected with 45 µg of proviral DNAs, and on the next day, M4-8 cells (10^6) were added to each culture. RT production in the cocultures was monitored at the designated intervals. Symbols: open circles, PBMCs transfected with pGH123 (wt); open triangles, pGH-St (vpx mutant); open squares, pGH-Ns (env mutant); closed circles, UV-killed PBMCs transfected with pGH123; closed triangles, pGH-St; closed squares, pGH-Ns.

![Time after coculture (days)](chart)

PBMC-A

PBMC-B

<table>
<thead>
<tr>
<th>Cells</th>
<th>Description &amp; Reference</th>
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<tbody>
<tr>
<td>CEM</td>
<td>human T-cell line, Foley et al (8)</td>
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<tr>
<td>CEMx174</td>
<td>human T-B hybrid cell line, Salter et al (27)</td>
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<td>human T-cell line, Shibata et al (28)</td>
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<tr>
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<td>U937</td>
<td>human histiocytic cell line, Ralph et al (23)</td>
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<tr>
<td>PBMC</td>
<td>primary culture of human blood cells, Ohta et al (22)</td>
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<tr>
<td>Macrophage</td>
<td>prepared from human PBMC, Gartner et al (10)</td>
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<sup>a</sup> Used by other research groups. Cell lines H9 and U937, and primary PBMCs were also used by other groups.
those previously reported (16). The late process was also examined by a transfection/coculture method (Fig. 5). Transfection of the wt and vpx-deficient proviral clones resulted in virus production in PBMCs with almost the same kinetics (Fig. 8), suggesting that the HIV-2 Vpx has no functional role in the late replication phase (from transcription to virion release). However, because of the assay method used here, the requirement of the Vpx protein at the late replication phase can not be ruled out.

The exact replication phase when the HIV-2 Vpx protein is required is still unknown. On the basis of the present functional studies and others (16), it can be concluded that the Vpx protein is necessary at an early stage(s) between adsorption/penetration and reverse transcription. The virion-associated nature of the Vpx protein is consistent with this conclusion. Of those early events in the viral life cycle, the process such as adsorption and penetration is unlikely to be mediated by the Vpx protein. However, this should be experimentally proved to determine the function of the HIV-2 Vpx protein.

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