Function of human immunodeficiency virus type 1 Vpu protein in various cell types

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We evaluated the function of human immunodeficiency virus type 1 vpu gene in various cell lines. We established a highly sensitive system consisting of chloramphenicol acetyltransferase and reverse transcriptase assays and used it to monitor the effects of mutation of the vpu gene. In some cell lines, Vpu protein was not required at the early phase of viral replication but was important for efficient virion production. In these cells, the Vpu protein functioned effectively irrespective of the presence of intact env gene products. Likewise, CD4 gene expression had no effects on Vpu function. In the other cell lines tested, Vpu protein was not important for virion release, and the vpu mutant clone generated a normal level of progeny virions upon transfection.

Introduction

Human immunodeficiency virus type 1 (HIV-1) has at least six accessory genes in addition to the gag, pol and env genes commonly found in retroviruses; this complexity is a characteristic of the lentivirus subgroup (Cullen & Greene, 1990). Investigation of their functions requires detailed comprehension of HIV-1 biology. Among these additional genes, vpu is unique to HIV-1, having no counterpart in the genomes of the closely related primate lentiviruses, which include HIV-2 and simian immunodeficiency viruses (SIVs). The vpu gene product is a 16 kDa phosphorylated protein (Cohen et al., 1988; Matsuda et al., 1988; Strebel et al., 1988) and its amino acid sequence has structural similarity to the membrane-associated viral protein (Strebel et al., 1988, 1989). Study of the growth kinetics of vpu-minus mutant viruses showed that the Vpu protein enhances the assembly and release of progeny virions from infected cells (Klimkait et al., 1990; Strebel et al., 1988, 1989; Terwilliger et al., 1989a). The finding that Vpu and Env proteins are translated from a single bicistronic mRNA indicates that coordinate expression of the vpu and env genes is required for efficient virus maturation (Arrigo & Chen, 1991; Schwartz et al., 1990).

In this report, we showed by means of a highly sensitive single-round infection assay, that the Vpu protein is required for efficient virus release in some cell types and that it does not alter the infectivity of the progeny virions. We also demonstrated that Vpu protein function is independent of env or CD4 gene expression.

Methods

Cell culture and DNA transfection. Adherent cell lines, J111 (JCRB0073; Japanese Cancer Research Resources Bank), IMR32 (ATCC CCL127), NB-1 (JCRB0621), T98G (ATCC CRL1690), RD (ATCC CCL136), HeLa (ATCC CCL 2), HeLa-CD4 designated as HT4-6C (CD4-positive HeLa cell line; Chesebro & Wehrly, 1988), SW480 (ATCC CCL228), COS-7 (ATCC CRL1651), AK-D (ATCC CCL150), Sirc (ATCC CCL60) and NIH3T3 (ATCC CRL1658) were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum (FCS). Non-adherent CD4-positive human T cell lines, A3.01 (Folks et al., 1985), H9 (Popovic et al., 1984), M8166 (Shibata et al., 1991), Molt4 (ATCC CRL1582) and SupT1 (Koenig et al., 1989) were maintained in RPMI1640 medium containing 10% heat-inactivated FCS. For transfection, uncleaved plasmid DNA was introduced into adherent and non-adherent cells by the calcium-phosphate coprecipitation (Graham & van der Eb, 1973; Wigler et al., 1979) and modified DEAE-dextran (Takai & Ohmori, 1990) methods.

Infection. The infectivity of progeny virions in transfected cells was assayed in A3.01 and H9 cells. Culture supernatants were filtered (pore size, 0.45 μm) and appropriate volumes were added to 10⁴ cells as described (Folks et al., 1985).

RT assay. Virion-associated reverse transcriptase (RT) activity was measured as described (Willey et al., 1988). Spots on DE81 paper (Whatman) were excised and RT activity was quantified by scintillation counting.

CAT assay. Chloramphenicol acetyltransferase (CAT) was assayed as described (Gorman et al., 1982) in equivalent amounts of cell lysates prepared from transfected or infected cells.

Immunoprecipitation analysis. A3.01 cells (2 x 10⁶) were transfected with 10 μg of proviral DNA and 20 μg of prevl (Sakai et al., 1990).

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Twenty-four h later, the cells were labelled for 20 h with [35S]methionine and immunoprecipitated (Sambrook et al., 1989), using a human anti-HIV-1 serum (ENVACOR HIV1 EIA kit; Abbott Laboratories). Sample lysates were prepared from cell pellets and the virion fraction. Precipitated proteins were resolved on 9–13% SDS–polyacrylamide gels and visualized by autoradiography.

**DNA constructs.** An infectious proviral clone of HIV-1 and its mutant, designated pNL432 (GenBank accession number M19921) and pNL-Ss (vpu mutant) have been described (Adachi et al., 1986, 1991; Ogawa et al., 1989). The pNLnCAT constructs were generated as described (Sakai et al., 1993a, b). Briefly, the CAT coding sequence of pSV2CAT (784 bp HindIII–Sau3AI fragment) (Sakai et al., 1990) was inserted between the XhoI (nt 8887) and KpnI (nt 9005) sites in the nef coding region of parental proviral clones and the rev coding sequence was inactivated by inserting an 8 bp XbaI linker (Toyobo) into the BamHI site (nt 8465). Plasmids pNLnCAT-SsNh and pNLnCAT-SsKp were generated from pNLnCAT-Ss (Fig. 2a) by digestion with NheI or KpnI, respectively, blunt-ended with T4 DNA polymerase, and resealed with T4 DNA ligase (Fig. 2a). Plasmids pNLnCATrc and pNLnCATrc-Ss, which produce replication-competent virus upon transfection, had identical structures to those of pNLnCAT and pNLnCAT-Ss, respectively, except for the absence of the XhoI linker insertion at the BamHI site. Plasmids pVpu and pVpu-m were constructed by inserting the vpu coding sequence (RsaI–KpnI, nt 6052–6343 fragment) of pNL432 and pNL-Ss, respectively, into the expression vector pRVSV (Sakai et al., 1990). The rev expression plasmid, prevl, has been described (Sakai et al., 1990).

**Results**

**Growth of vpu mutant viruses in CD4-positive cells**

When inoculated into CD4-positive cell lines, vpu-minus mutant viruses display unique and characteristic growth curves (Strebel et al., 1988; Terwilliger et al., 1989). We examined the growth properties of a vpu mutant virus derived from transfection of plasmid clone pNL-Ss (Adachi et al., 1991; Ogawa et al., 1989) in two CD4-positive cell lines. As shown in Fig. 1, the vpu mutant virus (NL-Ss virus) showed typical growth characteristics in both A3.01 and H9 cell lines. The growth kinetics of the NL-Ss virus were similar to those of wild-type (wt) virus (NL432 virus) but the levels of the NL-Ss virus in culture media were lower than that of the wt NL432 virus judging by RT levels. Viral cytopathic effect (CPE) was readily observed in both cell lines infected with the vpu mutant virus (data not shown). These results were reproduced in several independent experiments (data not shown). To determine the spread rate of the vpu mutant virus between cells, we generated the proviral clones, pNLnCATrc and pNLnCATrc-S (Fig. 2a), carrying a marker CAT gene and with structures similar to those of the pHXB-CAT constructs (Terwilliger et al., 1989b). Cell-free virus was prepared from transfected SW480 cells, and inoculated into A3.01 cells. When virus replication was monitored by RT production (Fig. 3) the vpu mutant virus displayed the typical growth characteristics shown in Fig. 1. In contrast, when CAT gene expression was used as a marker of virus replication, the vpu mutant virus grew quite well (Fig. 3).

**Analysis of replication cycle of vpu mutant clones**

It is, in general, technically difficult to quantify defective steps in the life cycle of growing retroviral mutants. To analyse the growth potential of the vpu mutant virus precisely, we developed a single-round infection assay (Sakai et al., 1993a, b), which is similar to a previously described system (Helseth et al., 1990). As shown in Fig. 2, this assay uses replication-defective rev mutant proviral clones carrying a bacterial CAT gene in the viral nef gene (Fig. 2a) and it is dependent upon trans-complementation for production of infectious vector virus (Fig. 2b). Simply by monitoring CAT and RT activities, the infection process can be estimated without background 'noise' originating from multiple rounds of replication.

To determine the infection stage at which Vpu is required, we constructed a vpu mutant clone of wt...
Functionality of HIV-1 Vpu

Fig. 2. A single-round infection assay. (a) Plasmid pNLnCAT constructs used in the assay. Restriction sites where mutations were introduced to generate a series of pNLnCAT constructs are indicated. To inactivate the rev gene, a stop codon linker was inserted into the BamHI site of the constructs. Plasmid clones with an intact rev gene, pNLnCATrc (wt) and pNLnCATrc-Ss (vpu mutant) were also constructed for infection studies (Fig. 3). Mutated genes in each pNLnCAT construct are summarized: +, wt; −, disrupted by a frameshift mutation; CAT, CAT gene in place of the wt nef gene. (b) Schematic representation of a single-round infection assay. Virions recovered from cells cotransfected with a replication-defective pNLnCAT construct (a) and a Rev-expression vector prev1 (Sakai et al., 1990) can undergo one round of replication in CD4-positive cells. CAT and RT production in transfected cells is indicative of a normal late replication phase (from transcription to virion release). The early phase (from attachment to integration) of virus replication can be monitored by the highly sensitive CAT assay.

pNLnCAT (designated pNLnCAT-Ss) (Fig. 2a) and monitored marker gene expression by means of a transfection (late phase)/infection (early phase) assay (Fig. 2b). One env and two vpu/env double mutants (Fig. 2a) were also constructed to evaluate the function of the Vpu protein without the expression of an intact env gene. As shown in Table 1, upon transfection into A3.01 cells, the vpu and vpu/env mutants expressed CAT activity similar to that of the wt clone. However, the amount of progeny released into the culture medium was significantly reduced relative to that of the wt clone. Immunoprecipitation demonstrated 3-4-fold more viral Gag p24 within cells transfected with the vpu mutant than in those transfected with the wt clone but the opposite was true of the virion fraction (Fig. 4). Consistent with these results, the vpu mutant induced syncytia in transfected A3.01 cells (data not shown) showing the expression of authentic env gene products on the cell surface (Freed et al., 1989). To determine whether the early infection phase of the vpu mutant virus proceeded normally, progeny virions derived from transfected A3.01 cells were inoculated onto fresh A3.01 cells and CAT activity was monitored (Table 1). There was no significant difference between CAT activities expressed by wt and the vpu mutant.

**Functionality of the Vpu protein in various cell lines**

We found that the vpu mutant exhibited the wt phenotype in transfected SW480 cells and that its progeny derived from SW480 cells also had normal infectivity in A3.01 cells in a single-round infection assay (Sakai et al., 1993 a). To confirm this cell-type specificity, we examined virion production by the vpu mutant in a wide variety of cell lines (Table 2). Proviral clones were transfected into cells and 48 h later, RT activity in the culture media was measured. RT levels in the culture media of the different cell types transfected with the proviral clones varied, but the ratio of RT production between the vpu mutant and wt clones in each cell line was constant in two independent experiments (Table 2). Therefore, the values presented in Table 2 were indicative of the cell-type dependence of Vpu expression for efficient virus release. In many cell lines, mutation in the viral vpu gene greatly reduced virion production. Among the leukaemia cell lines tested, SupT1 cells released a relatively high level of
Fig. 3. Growth kinetics of a *vpu* mutant virus carrying the CAT gene in CD4-positive A3.01 cells. A3.01 cells ($10^5$) were infected with cell-free viruses obtained from SW480 cells transfected with pNLnCATrc or pNLnCATrc-Ss ($1 \times 10^6$ RT units). RT production in culture medium was monitored at intervals (upper panel). At the same time, cell lysates were prepared, and CAT activity was determined (lower panel). Symbols: $\bigcirc$, virus from pNLnCATrc (wt); $\triangle$, virus from pNLnCATrc-Ss (*vpu* mutant); $\square$, mock-infection.

Table 1. Marker gene expression of a *vpu* gene mutant in CD4-positive A3.01 cells*

<table>
<thead>
<tr>
<th>Clones</th>
<th>Transfection</th>
<th>Infection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CAT activity (%)</td>
<td>RT activity (%)</td>
</tr>
<tr>
<td>pNLnCAT (none)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>pNLnCAT-Ss (<em>vpu</em>)</td>
<td>98</td>
<td>22</td>
</tr>
<tr>
<td>pNLnCAT-Nh (env)</td>
<td>105</td>
<td>110</td>
</tr>
<tr>
<td>pNLnCAT-SsKp (<em>vpu-env</em>)</td>
<td>102</td>
<td>50</td>
</tr>
<tr>
<td>pNLnCAT-SsNh (vpu-env)</td>
<td>110</td>
<td>23</td>
</tr>
</tbody>
</table>

* Activity relative to that of the wt clone is presented. A3.01 cells were cotransfected with one of the pNLnCAT constructs (Fig. 2a; 10 µg) and prev1 (20 µg). Two days later, CAT activity in the cell lysate and RT activity in culture fluids were determined as described (Gorman et al., 1982; Willey et al., 1988). Culture supernatants harvested from transfected A3.01 cells were filtered (0.45 µm pore size), adjusted to $10^7$ RT units and used for inoculation. CAT activity in the cell lysates was determined two days after infection. For plasmid designations, see Fig. 2. Mutated genes are indicated in parentheses.

Fig. 4. Immunoprecipitation analysis of Gag and Env proteins of a *vpu* mutant. Two fractions were analysed by immunoprecipitation. These were cell lysate (total cell lysate) and virion (ultracentrifugation pellet). A3.01 cells were cotransfected with prev1 (20 µg) and pNLnCAT (wt) or pNLnCAT-Ss (*vpu* mutant) (10 µg). Twenty-four h later, the cells were labelled with [35S]methionine and immunoprecipitated (Sambrook et al., 1989) using a human anti-HIV-1 serum. Env protein in the virions obtained from transfection was difficult to detect in repeated experiments.

virions after transfection with the *vpu* mutant. Of the adherent cell lines tested, T98G, HeLa, HeLa-CD4 and Sirc cells produced a relatively low level of virions in the absence of an intact *vpu* gene. In IMR32, NB-1, SW480, COS-7 and NIH3T3 cells, the *vpu* mutant expressed normal levels of progeny upon transfection.

To determine whether the Vpu-dependent cell lines lack some factor(s) equivalent to the Vpu protein required for efficient virion production, we performed trans-complementation studies using a Vpu-expression vector. As shown in Fig. 5, cotransfection of the Vpu-expression vector significantly increased the amount of progeny virions of the *vpu* mutant clone in the Vpu-dependent cell line A3.01. This effect was not observed in the Vpu-independent cell line SW480. Furthermore, a mutation in the *vpu* gene of the expression vector abolished this enhancing effect.

**Discussion**

The major finding of this study was that the HIV-1 Vpu protein is required, in a cell-specific manner, for efficient virion release. Of 17 cell lines tested, five produced progeny virions normally when transfected with the *vpu*...
Table 2. Virion production in various cell lines transfected with pNLnCAT constructs*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Relative RT production (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3.01</td>
<td>Human CD4+ leukaemia cells</td>
<td>0.2</td>
</tr>
<tr>
<td>H9</td>
<td>Human CD4+ leukaemia cells</td>
<td>0.2</td>
</tr>
<tr>
<td>M8166</td>
<td>Human CD4+ leukaemia cells</td>
<td>0.1</td>
</tr>
<tr>
<td>MolT4</td>
<td>Human CD4+ leukaemia cells</td>
<td>0.4</td>
</tr>
<tr>
<td>SupT1</td>
<td>Human CD4+ leukaemia cells</td>
<td>0.7</td>
</tr>
<tr>
<td>Jlll</td>
<td>Human monocytic leukaemia cells</td>
<td>0.2</td>
</tr>
<tr>
<td>IMR32</td>
<td>Human neuroblastoma cells</td>
<td>1.2</td>
</tr>
<tr>
<td>NB-1</td>
<td>Human neuroblastoma cells</td>
<td>1.0</td>
</tr>
<tr>
<td>T98G</td>
<td>Human glioblastoma cells</td>
<td>0.3</td>
</tr>
<tr>
<td>RD</td>
<td>Human rhabdomyosarcoma cells</td>
<td>0.6</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma cells</td>
<td>0.3</td>
</tr>
<tr>
<td>HeLa-CD4</td>
<td>CD4+ HeLa cells</td>
<td>0.3</td>
</tr>
<tr>
<td>SW480</td>
<td>Human colon carcinoma cells</td>
<td>10</td>
</tr>
<tr>
<td>COS-7</td>
<td>Monkey kidney cells</td>
<td>10</td>
</tr>
<tr>
<td>AK-D</td>
<td>Cat lung cells</td>
<td>0.7</td>
</tr>
<tr>
<td>Sirc</td>
<td>Rabbit corneal cells</td>
<td>0.3</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Mouse fibroblast cells</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*RT production in transfected cells relative to that of the wt clone is shown. Cells were cotransfected with pNLnCAT or pNLnCAT-Ss (Fig. 2a; 10 µg) and prevl (20 µg). Two days later, RT activity in culture fluids was determined as described (Willey et al., 1988). Values represent relative titres (RT activity by pNLnCAT-Ss/RT activity by pNLnCAT). The efficiency of transfection between the mutant and wt clones was almost equal, as monitored by CAT assays. Two independent experiments were performed with similar results (less than 10% variation).

minus mutant clone. In Vpu-dependent cell lines, the vpu-minus mutant clones were relatively poor producers of progeny upon transfection. We demonstrated by the single-round infection assay that the Vpu protein of HIV-1 has no functional role in viral attachment or in the early stages of infection up to viral protein synthesis. Instead, the Vpu protein is important for virion assembly/maturation as previously described (Klimkait et al., 1990; Strebel et al., 1988, 1989; Terwilliger et al., 1989a, Yao et al., 1992). It acted in trans to enhance virion production in the Vpu-dependent cells. For function of the Vpu protein in these cells, expression of the CD4 gene or viral env gene is not necessary, consistent with the reported results (Yao et al., 1992). The Vpu protein regulates the formation of intracellular gp160–CD4 complexes (Willey et al., 1992). The molecular basis for the Vpu action remains to be clarified.

From a virological perspective, it is notable that the vpu mutant viruses display a unique growth property. Although there were no significant differences in infectivity between wt and the vpu mutant virions, a relatively low number of virus particles were released into the culture medium from cells infected with the mutant viruses. These results indicated that the similar virus transmission rate between wt and the vpu mutant virus shown in Fig. 3 can be accounted for by a more rapid cell-to-cell spread of the vpu mutant. All primate immunodeficiency viruses other than those of the HIV-1 group lack the vpu gene. They easily establish a spreading and productive infection in various target cells.

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References


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