Expression and processing of human immunodeficiency virus type 1 gp160 using the vesicular stomatitis virus New Jersey serotype vector system

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The Indiana serotype of vesicular stomatitis virus (VSVIND), but not the New Jersey serotype (VSVNJ), has been widely used as a gene expression vector. In terms of prime–boost-based vaccine strategies, it would be desirable to use two different VSV serotypes to avoid immunity against the priming viral vector. Here, we report that we have applied the VSVNJ vector system for expression of the env gene of human immunodeficiency virus type 1 (HIV-1). The HIV-1 env gene was inserted into the VSVNJ vector system at two different sites: between the P and M genes (NP-gp160-MGL) and between the G and L genes (NPMG-gp160-L). The HIV-1 env gene product, gp160, was efficiently expressed and processed in cells infected with either of these two recombinant VSV–HIV-1gp160 viruses. In this study, we have investigated the applicability of the VSVNJ vector system for foreign gene expression.

Vesicular stomatitis virus (VSV) is the prototypical member of the family Rhabdoviridae, it is a non-segmented, negative-sense, RNA virus. VSV causes vesicular stomatitis in cattle, horses and swine. Usually, VSV infection in humans is asymptomatic or causes influenza-like symptoms, i.e. fever, malaise, etc. (Fields & Hawkins, 1966). Based on its serological cross-reactivity patterns, VSV is divided into two major serotypes, Indiana and New Jersey (VSVIND and VSVNJ, respectively).

The VSV genome contains five protein-coding genes which are in the following order: 3′-N-P-M-G-L-5′ (Abraham & Banerjee, 1976). There are conserved, untranslated, intergenic junctions between each gene, which contain the transcription termination and polyadenylation signals for the upstream gene and the transcription reinitiation signals for the subsequent downstream gene (Rose, 1980; Rose & Schubert, 1987; Schnell et al., 1996b). In terms of transcriptional attenuation of the VSV genome, the quantity of transcripts produced from each of the downstream genes is approximately 30% lower than those of the gene located immediately upstream (Villarreal et al., 1976).

Since a reverse genetics system for the recovery of recombinant VSV from cDNA plasmids was first established in 1995 (Lawson et al., 1995), VSV has been widely used as a viral vector system for foreign gene expression and vaccine development. Recombinant VSV expressing the haemagglutinin protein of influenza virus generates a strong protective immune response in mice (Roberts et al., 1998, 1999); a number of vaccine candidates using recombinant VSV vectors have been designed and tested in animal models to protect against infectious diseases such as AIDS, hepatitis, severe acute respiratory syndrome and others (Egan et al., 2004; Kapadia et al., 2005; McGettigan et al., 2003; McKenna et al., 2003; Rose et al., 2001).

Until now, all VSV-based gene expression experiments have been carried out using VSVIND. Although it is believed that the New Jersey serotype has the same potential as the Indiana serotype, VSVNJ has not been reported as a gene expression vector. In terms of prime–boost-based vaccine strategies, it is highly desirable to use both serotypes of VSV to express the foreign antigen, so that the immune response elicited by one serotype of VSV vector during the priming vaccination will not result in the generation of neutralizing antibodies against the boosting VSV (Rose et al., 2000, 2001). The G protein of VSV is the major antigenic target for neutralizing antibody responses and the G protein amino acid sequences of the Indiana and New Jersey serotypes are considerably different. Thus, the cross-neutralization between these two serotypes is very low (Kelley et al., 1972). Utilizing the two serotypes of VSV as vectors in a prime–boost regimen may, therefore, avoid potential vector-based vaccine rejection. For this reason, we established the VSVNJ vector system (Kim & Kang, 2007). To generate this vector, the full-length VSVNJ genome was inserted into the backbone transcription
vector, pTV (provided by Dr A. Ball, University of Alabama, Birmingham, USA), downstream of the T7 promoter, so that the genome could be transcribed by T7 RNA polymerase. The mRNA transcripts could be cut at the end of the trailer sequence by the hepatitis delta virus ribozyme, which is inserted immediately downstream of the VSV genome, so that the genomic RNA is identical to that produced by natural infection.

To test whether the VSVNJ vector system has the ability to express and process the foreign glycoprotein, we used it to express the HIV-1 env gene. The HIV-1 Env glycoprotein gp160 precursor is synthesized and glycosylated in the rough endoplasmic reticulum (ER) and then transported into the Golgi, where it is cleaved into gp120 and gp41 by cellular proteases (Moulard & Decroly, 2000; Stein & Engleman, 1990). gp120 and gp41 remain associated via noncovalent, intramolecular bonds and are transported from the Golgi to the cell surface. Since this noncovalent association is very weak, gp120 is fairly easily shed from the cell surface into the culture medium (Schneider et al., 1986).

Due to transcription attenuation, the foreign gene can be more efficiently expressed if it is inserted closer to the leader. However, the position of the N gene is critical for viral replication since an adequate quantity of N protein is important for initiation of replication of the RNA genome (Flanagan et al., 2000; Wertz et al., 1998). Therefore, it is better to leave the N gene at the first position immediately following the leader. The molar ratio of the N and P proteins is critical for both transcription and replication of VSV, thus insertion of foreign genes between the N and P genes decreases viral replication by 10- to 30-fold compared with the wild-type virus (Wertz et al., 2002). Therefore, the positions between the P and M genes and between the G and L genes were selected as sites for the insertion of HIV-1 gp160 in this study.

We generated two full-length cDNA clones of VSVNJ containing the HIV-1 env gene inserted at two different positions: between the P and M genes and between the G and L genes (Fig. 1a). The recombinant VSV-HIV-1gp160 viruses, NP-gp160-MGL and NPMG-gp160-L, as well as the recombinant VSVNJ wild-type (rVSVNJ wt) were recovered by reverse genetics (see Supplementary Methods, available in JGV Online). To confirm whether the recombinant VSV-HIV-1gp160 viruses contain the env gene in their genomes, viral RNA was isolated from recombinant VSV-HIV-1gp160 and RT-PCR was performed. The RT-PCR products were analysed by DNA sequencing analysis, which confirmed that the correct HIV-1 env gene was inserted in the expected positions (data not shown).

A growth curve was constructed to examine the growth kinetics of the recombinant VSVs compared with wild-type VSV (VSVNJ wt). BHK-21 cells were infected with NP-gp160-MGL, NPMG-gp160-L, rVSVNJ wt and VSVNJ wt at an m.o.i. of 3. The infected cell culture supernatants were collected every hour from 0 to 10 h post-infection (p.i.).

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Fig. 1. gp160 expression in cells infected with recombinant VSV-HIV-1gp160 viruses. (a) Schematic diagrams of pTV-VSVNJ, pTV-NP-gp160-MGL and pTV-NPMG-gp160-L. L, leader; t, trailer; T7P, T7 RNA polymerase promoter; H, hepatitis delta virus ribozyme. (b) Growth curves of recombinant VSV-HIV-1gp160 viruses. Data shown are means ± SEM. (c) Detection of gp160 expression in cells infected with recombinant VSV-HIV-1gp160 viruses by [35S]methionine labelling. Lanes: 1, NP-gp160-MGL; 2, NPMG-gp160-L; 3, rVSVNJ wt.
The viral titres of samples were determined by plaque assay. The results showed that the replication patterns of the recombinant viruses were similar to those of the wild-type viruses (Fig. 1b). The final titres of the recombinant VSVs expressing HIV-1 gp160 were approximately 10-fold lower than those of rVSVNJ wt and VSVNJ wt. The NP-gp160-MGL replicated slightly slower than NPMG-gp160-L and the final titre of NP-gp160-MGL was about twofold lower than NPMG-gp160-L. Our results show clearly that the VSVNJ genome can accommodate foreign genes without a significant reduction in VSV replication.

To examine gp160 expression in recombinant VSV-infected cells, BHK-21 cells were infected with recombinant VSVs at an m.o.i. of 6 and labelled with 50 μCi (1.85 MBq) [35S]methionine ml⁻¹ for 1 h. At 4 h p.i., cells were harvested and lysed, and total cell lysates were analysed by SDS-PAGE and autoradiography. The rVSVNJ wt served as a negative control. In order to separate gp160 from VSV L protein, electrophoresis was carried out for a much longer time. Thus, the VSV M protein ran off the gel. The lysates of both NP-gp160-MGL- and NPMG-gp160-L-infected cells showed additional bands of the sizes expected for gp160, while those infected with rVSVNJ wt did not show the same band (Fig. 1c). This suggests that both recombinant viruses containing the HIV-1 env gene inserted between either the P and M or the G and L genes expressed gp160 efficiently. However, NP-gp160-MGL, which was expected to express gp160 more efficiently, did not show a higher level of gp160 expression than NPMG-gp160-L.

To determine whether gp160 can be processed properly in cells infected with recombinant VSV-HIV-1gp160 viruses, we analysed both gp120 and gp41 in infected cells and in culture supernatants. BHK-21 cells were infected with recombinant VSVs at an m.o.i. of 6. At 6 h p.i., both infected cells and cell culture supernatants were collected and analysed by Western blot analysis using goat anti-HIV-1 gp120 polyclonal antibody (BIODESIGN). rVSVNJ wt served as a negative control. The results showed that gp120 appeared both in infected cell lysates and in infected culture supernatants (Fig. 2a). In addition, the amount of unprocessed gp160 found in the cell was much higher than the amount of processed gp120. In contrast, only gp120 was detected in the culture supernatant as expected. This demonstrated that only processed gp120 was released from the cells. Another cleavage product of gp160, gp41, was also analysed by Western blot, using a human anti-HIV-1 gp41 monoclonal antibody (Dr M. Posner, NIH). gp41 was found in both the infected cells and the cell culture supernatant (Fig. 2b). To examine whether gp120 and/or gp41 are incorporated into the progeny virions of the recombinant VSVs, newly synthesized virus particles were

![Fig. 2. Expression and processing of gp160 produced by infection with recombinant VSV-HIV-1gp160 viruses. (a) Detection of gp120 in infected cell lysates (left) and infected cell culture supernatant (right). (b) Detection of gp41 in infected cell lysates (left) and infected cell culture supernatant (right). (c) Detection of gp120 (left) and gp41 (right) in purified viral particles. Lanes: 1, NP-gp160-MGL; 2, NPMG-gp160-L; 3, rVSVNJ wt; 4, uninfected cell. Size markers are shown (kDa).](http://vir.sgmjournals.org)
pelleted, purified and analysed by SDS-PAGE followed by Western blot. We found that both NP-gp160-MGL and NPMG-gp160-L packaged gp41 into the virions. In contrast, gp120 was not detected in the recombinant VSV-HIV-1gp160 viruses (Fig. 2c). These results demonstrate that gp41 is incorporated into the recombinant VSV particles but gp120 is not; the reason for this is not clear. The association between gp120 and gp41 is rather weak; thus, gp120 might have been dissociated from gp41 during virus particle purification. The incorporation of HIV-1 gp120 into VSVIND particles is also relatively low (Johnson et al., 1997; Schnell et al., 1996a).

Pulse–chase analyses were carried out to study the kinetics of gp160 processing in cells infected with the recombinant VSV-HIV-1gp160 viruses further. Briefly, BHK-21 cells were infected with recombinant VSVs at an m.o.i. of 6, pulse-labelled with [35S]methionine for 1 h at 4 h p.i. and chased with fresh culture medium for 0, 1, 2, 3 and 4 h. Both cell lysates and culture supernatants were immunoprecipitated with a goat anti-HIV-1 gp120 polyclonal antibody, which can recognize both gp160 and gp120, and analysed by SDS-PAGE. The results showed that gp160 was present in cell lysates and gradually decreased during the chase period. Meanwhile, the amount of gp120 in the culture medium gradually increased (Fig. 3). This demonstrates that gp160 was processed in cells infected with both NP-gp160-MGL and NPMG-gp160-L.

In this study, we have presented the applicability of the VSVNj vector system for foreign gene expression. We have inserted the HIV-1 env gene into two different sites in the VSVNj genome (between the P and M genes and between the G and L genes) and recovered recombinant VSV-HIV-1gp160 viruses (NP-gp160-MGL and NPMG-gp160-L, respectively) by using the VSV reverse genetics system (Kim & Kang, 2007). We demonstrated that the VSVNj vector can tolerate a large foreign gene insertion at a position between either the P and M or G and L genes. The recovery of recombinant VSV-HIV-1gp160 viruses was slower than that of the rVSV wt and the titres of the recombinant VSV-HIV-1gp160 viruses were 10-fold lower than those of VSVNj wt. This difference may be caused by the large insertion in the VSV genome.

The results presented here demonstrate that both recombinant viruses can produce gp160 efficiently. Unexpectedly, NP-gp160-MGL did not show a higher expression level of gp160 compared with NPMG-gp160-L. The one-step growth curve showed that the replication rate of NP-gp160-MGL was slightly lower than NPMG-gp160-L and the final titre of the NP-gp160-MGL was also twofold lower than NPMG-gp160-L. This might be the reason that the NP-gp160-MGL did not express gp160 as efficiently as we expected.

In the recombinant VSV-HIV-1gp160 virus-infected cells, the HIV-1 env gene was expressed and the precursor gp160 protein was cleaved. gp160, gp120 and gp41 were detected in the infected cell; however, the amount of gp120 released into the supernatant of cells infected with either NP-gp160-MGL or NPMG-gp160-L was of a relatively small quantity compared with the large amount of gp160 expressed in the infected cells. Due to the ER quality control system, gp160 must be correctly and completely folded before being allowed to leave the ER for further transport to the Golgi, where it is cleaved by cellular protease. The relatively high proportion of unfolded and misfolded gp160 is associated with calnexin and remains in the ER (Ellgaard et al., 1999; Ellis & Hemmingsen, 1989). Thus, the quantity of gp120 is lower than gp160. This low level of gp120 may also have been caused by the VSV M protein, which is largely responsible for the cytopathic effects associated with VSV infection (Blondel et al., 1990; Melki et al., 1994). During VSV infection, the host cell protein synthesis is shut down by M protein (Black et al., 1993, 1994; Black & Lyles, 1992). Therefore, there may not be enough cellular protease to cleave gp160 into gp120 and gp41. Another reason for the inefficient release of gp120 may be the internalization of the gp120–gp41 complex. After the gp120–gp41 complex is transported to the cell surface, it is rapidly internalized, especially when the Gag 55 kDa polypeptide precursor is absent (Egan et al., 1996; LaBranche et al., 1995; Rowell et al., 1995).

As mentioned above, the M protein of VSV can shut down the cellular protein synthesis of the host cells and induce cytopathic effects. In order to avoid this problem, the
replication-competent VSV_{NJ} vector with mutations in the M protein has been constructed as described here. Studies to evaluate the ability of foreign gene expression by this new vector are in progress.

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