Increased human immunodeficiency virus type 1 Env expression and antibody induction using an enhanced alphavirus vector

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Viral vectors encoding heterologous vaccine antigens are potent inducers of cellular immune responses, but they are generally less efficient at stimulating humoral immunity. To improve the induction of antibody responses by Semliki Forest virus-based vaccines, a vector encoding a translation-enhancer element and a novel internal signal sequence for increased expression and secretion of soluble antigens was designed. Approximately tenfold more human immunodeficiency virus type 1 gp120 was secreted into culture supernatants of infected cells using the enhanced vector compared with the parental vector. This translated into a significant increase in gp120-specific antibodies in immunized mice, suggesting that antigen-expression levels from the parental vector are limiting for induction of antibody responses. These data encourage the use of the enhanced vector for elicitation of immune responses against heterologous antigens during vaccination.

Recombinant viral vectors have the potential to induce both humoral and cellular immune responses and they may therefore have value in the development of vaccines against viruses such as human immunodeficiency virus type 1 (HIV-1). Single-round infectious recombinant Semliki Forest virus (rSFV) vaccine vectors have been shown to induce potent cellular responses against a variety of foreign antigens in small animals and in non-human primates (Berglund et al., 1997, 1999; Fleeton et al., 1999). However, like many other viral vectors, rSFV-based vaccines are relatively poor at inducing humoral immunity in the absence of subsequent booster immunizations with purified protein antigen (Forsell et al., 2005). Heterologous regimens based on priming with viral vectors and boosting with purified protein therefore represent an attractive means to induce both cellular and humoral immune responses (Lubeck et al., 1997; Malkevitch & Robert-Guroff, 2004; Montefiori et al., 1992; Patterson et al., 2004; Shu et al., 2006).

We showed recently that rSFV vectors can drive the expression of soluble HIV-1 envelope glycoprotein (Env) monomers and trimers and that these molecules are recognized by conformation-sensitive antibodies, suggesting that native folding is retained (Forsell et al., 2005). We also showed that rSFV-Env vectors prime Env-directed antibody responses efficiently when followed by a boost with purified matched Env protein antigen (Forsell et al., 2005). One likely explanation for the need of a protein boost is that the amount of antigen produced by replication-defective viral vectors is suboptimal. A vector designed to combine viral adjuvant properties with the ability to elicit cellular responses and to produce high levels of B-cell antigens would therefore be desirable and may reduce the need for subsequent protein boosts. To produce such a vector and to investigate the effect of antigen-expression levels for antibody elicitation, we designed a novel rSFV vector, rSFV-Eiss, that encodes the SFV translation-enhancer element (Sjoberg et al., 1994) upstream of and in frame with an internal signal sequence (iss) to drive the secretion of soluble HIV-1 gp120. The enhancer element has previously been shown to promote translation of downstream sequences under highly restrictive conditions, such as during rSFV-induced host-cell translational shut-off (McInerney et al., 2005; Sjoberg et al., 1994). However, it promotes the increased expression of heterologous antigens only when situated upstream of and in frame with those antigens (Sjoberg et al., 1994), thus preventing the use of standard N-terminal signal sequences to direct the antigen into the endoplasmic reticulum (ER). To overcome this obstacle, we constructed a vector encoding an iss placed downstream of the enhancer element (E) and upstream of gp120. This way, the increased expression provided by the translation-enhancer element is combined with a mechanism allowing native gp120 to be processed...
correctly in the ER membrane prior to its secretion. Specifically, the rSFV-Eiss-gp120 vector was created by inserting the enhancer element, the first 103 nt of the SFV subgenomic RNA capsid-coding region, into the vector downstream of the subgenomic promoter. The iss, derived from the SFV E1 spike protein (Liljestrom & Garoff, 1991) (Fig. 1a), was inserted in frame between the regions encoding the enhancer and gp120 from the primary HIV-1 isolate YU2 (Fig. 1b) (Li et al., 1991).

The new vector was compared side by side with the standard rSFV vector for gp120 expression levels in vitro. The parental rSFV-gp120 vector contains a heterologous N-terminal signal sequence derived from CD5 (Fig. 1b), which is more efficient than the native HIV-1 Env signal sequence (Grundner et al., 2005). rSFV-gp120 and rSFV-Eiss-gp120 particles, prepared by using the rSFV split helper system (Smerdou & Liljestrom, 1999), were used to infect BHK-21 cells (ATCC) at an m.o.i. of 20. At 12 h post-infection (p.i.), the cells were incubated in methionine-free (starvation) medium for 15 min; they were then pulsed by incubation in starvation medium supplemented with 50 μCi (1.85 MBq) [35S]methionine ml⁻¹ for 15 min and chased in complete medium according to standard procedures (Karlsson & Liljestrom, 2004). At various chase times, supernatants from infected cells were collected, and radioactively labelled proteins were separated by SDS-PAGE and visualized by autoradiography. Different dilutions of the supernatant from rSFV-Eiss-gp120-infected cells are shown. (e) Western blot analysis of supernatants from rSFV-gp120- and rSFV-Eiss-gp120-infected BHK-21 cells harvested 12 h after infection. A polyclonal anti-gp120 serum was used for detection.
infected cultures, we diluted supernatants from cells infected with rSFV-Eiss-gp120 twofold, fivefold and tenfold and compared them with undiluted supernatant from rSFV-gp120-infected cells (Fig. 1d). Densitometry analysis of the bands (data not shown) demonstrated that approximately tenfold more gp120 was secreted by rSFV-Eiss-gp120-infected cells than by cells infected with rSFV-gp120 after a 240 min chase, consistent with the increase in expression of intracellular proteins reported previously from vectors encoding the SFV enhancer element (Berglund et al., 1998, 2007; Huckriede et al., 2004; Karlsson & Liljestrom, 2004; Sjoberg et al., 1994). To examine the difference in gp120 levels secreted by the two vectors by another method, we also analysed non-labelled supernatants harvested at 12 h p.i. for gp120 by using Western blot analysis. This experiment confirmed that rSFV-Eiss-gp120-infected cells produced considerably higher levels of gp120 than cells infected with rSFV-gp120 (Fig. 1e). Furthermore, N-terminal sequencing of the unlabelled secreted gp120 product from rSFV-Eiss-gp120-infected cells confirmed that proteolytic cleavage had occurred after the C-terminal Ala–Arg–Ala (ARA) signal peptidase cleavage motif present in the iss, creating the expected -GNLWVTYYG- N terminus of YU2gp120 (Fig. 1b).

To examine the biosynthesis of gp120 produced from the two vectors in more detail, we analysed the supernatants and cell lysates from an independent pulse–chase experiment after endoglycosidase H (Endo H; Roche) treatment of the labelled proteins. Whereas only one major protein form was detected in the untreated lysates from rSFV-gp120-infected cells (Fig. 2a), two major protein forms were detected in lysates from rSFV-Eiss-gp120-infected cells (Fig. 2b). The lower-mobility form was consistent with fully glycosylated gp120, whilst the higher-mobility form migrated with an apparent molecular mass of about 55 kDa, corresponding to non-glycosylated gp120. The presence of the 55 kDa form is likely because the cells are unable to direct the translocation of all overexpressed nascent polypeptides across the ER membrane. Endo H treatment of cell-associated proteins taken 15 or 60 min after chase indicated that most of gp120 was retained in an immature (high-mannose), fully Endo H-sensitive form, which migrated with an apparent molecular mass of 125 kDa. After 240 min chase, gp120 migrated as a 120 kDa protein, suggesting that some of the glycans had been processed to smaller complex-type oligosaccharides. At this time point, most of the gp120 had been transported into the supernatant and the secreted protein was partially Endo H-resistant, consistent with the presence of both high mannose- and complex-type oligosaccharides on mature gp120, as reported previously (Leonard et al., 1990; Sanders et al., 2002a) (Fig. 2a, right-hand panel). The kinetics of maturation and secretion of gp120 from rSFV-Eiss-gp120-infected cells were similar (Fig. 2b, right-hand panel), demonstrating that even when gp120 is highly overexpressed from the rSFV-Eiss vector, the secreted product retains a biosynthetically mature phenotype.

![Fig. 2. Biosynthesis of HIV-1 gp120 in rSFV-infected cells.](image)

BHK-21 cells, infected with (a) rSFV-gp120 or (b) rSFV-Eiss-gp120, were analysed for post-translational processing of gp120. Infected cultures were pulsed for 15 min with [35S]methionine and chased for 15, 60 or 240 min before lysates and supernatants were harvested. Samples were treated for 6 h with Endo H and the proteins were separated by SDS-PAGE and visualized by autoradiography. Immature, fully glycosylated high-mannose gp120 present in the lysates at the early time points migrates with an apparent molecular mass of approximately 125 kDa (●), whereas the Endo H-treated immature gp120 migrates with an apparent molecular mass of 55 kDa. After 240 min chase, some of the gp120 is mature and migrates with an apparent molecular mass of 120 kDa (◆), which is partially Endo H-sensitive (○). Most of the mature gp120 is found in the culture supernatant.

Having characterized the secreted gp120 produced from rSFV-Eiss-gp120- and rSFV-gp120-infected cells, we performed immunogenicity studies in mice to examine whether differences in expression levels would translate into differences in gp120-directed immune responses. Because the rSFV-gp120 and rSFV-Eiss-gp120 vectors in all other parts are identical, we could examine the effect of antigen levels without altering the number of virus particles used for immunization. This is important, as viral particles have been shown to possess intrinsic, dose-dependent adjuvant effects (Boudet et al., 2001; Brimnes et al., 2003; Hidmark et al., 2006; Hutchings et al., 2005; Thompson et al., 2006). BALB/c mice were immunized twice subcutaneously with 100 µl containing 1 × 10⁷ infectious units (IU) either rSFV-gp120 or rSFV-Eiss-gp120 in PBS, at an interval of 3 weeks. The mice were bled 12 days after the second immunization and the serum was analysed for anti-gp120 reactivity by using an ELISA, as described previously (Forsell et al., 2005). The results show that six of six BALB/
found that both vectors induced HIV-1 Env-specific IFN-γ ELISPOT analysis (Forsell et al., 2000, 2001). We have shown previously that rSFV vector encoding the influenza nucleocapsid protein were included as a negative control (×). gp120-specific ELISA end-point titres in sera from immunized mice. The end-point titre was defined as the last reciprocal serum dilution at which the mean –2SD of duplicate wells had an A<sub>450</sub> value >0.21 (representing the mean A<sub>450</sub> of sera from negative-control animals at a reciprocal serum dilution of 50). The dotted line indicates an end-point titre of 50. This represents the lowest reciprocal serum dilution at which gp120-specific responses above background could be determined in the ELISA assay. (c) CD4<sup>+</sup> T-cell ELISPOT analysis for IFN-γ secretion after in vitro stimulation with insect cell-produced gp120. Env-specific IFN-γ CD4<sup>+</sup> T-cell responses significantly higher (P<0.05) than control stimulations (medium alone) were detected in both rSFV-gp120-immunized mice (empty bars) and rSFV-Eiss-gp120-immunized mice (filled bars). There was no statistically significant difference in the magnitude of the Env-specific response between the two groups. The bars represent the means of five mice per group, with the error bars indicating SD. Unpaired t-test was used for statistics.

As differences in CD4<sup>+</sup> T-cell responses can affect antibody elicitation against T cell-dependent antigens such as Env, we next investigated CD4<sup>+</sup> T-cell responses in rSFV-Eiss-gp120- and rSFV-gp120-immunized mice after antigen stimulation of splenocytes as described previously (Forsell et al., 2005). We have shown previously that rSFV immunization stimulates a Th1-biased response, with detectable CD4<sup>+</sup> T-cell gamma interferon (IFN-γ) production upon antigen restimulation in vitro, but with no detectable production of interleukin-4, as determined by ELISpot analysis (Forsell et al., 2005). In this study, we found that both vectors induced HIV-1 Env-specific IFN-γ CD4<sup>+</sup> T-cell responses upon immunization and there was no significant difference in the magnitude of the response induced by the two vectors (Fig. 3c). This suggests that antigen levels produced by rSFV-gp120 are not limiting for induction of CD4<sup>+</sup> T-cell help. Further analysis of the quality of the immune responses induced by the enhanced rSFV-Eiss vector, including the ability of the vectors to elicit broadly neutralizing antibodies against HIV-1, will require the use of Env immunogens that mimic the functional viral spike better than the monomeric gp120 used here (Barnett et al., 2001; Binley et al., 2000; Earl et al., 1994, 2001; Farzan et al., 1998; Sanders et al., 2002b; Schulke et al., 2002; Yang et al., 2000, 2001, 2002). Thus, neutralizing-antibody responses were not analysed in this study.

In conclusion, we show that the rSFV vector system can be modified to encode a translation enhancer inserted in frame with an iss, allowing enhanced expression and secretion, respectively, of mature HIV-1 Env glycoproteins. When the enhanced vector was used to induce anti-Env antibody responses in mice, a significant improvement in antibody titres was observed compared with the responses elicited by the conventional rSFV vector. These data encourage the use of rSFV-Eiss to overcome some of the limitations of the rSFV vector system to induce humoral immune responses. Furthermore, the SFV enhancer element inserted in frame with the iss used here could also be used in some other well-selected viral vector systems to enhance secretion of soluble antigens. Thus, this vector design could...
be a more broadly applicable means to enhance immune responses.

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References


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