Envelope proteins of spleen necrosis virus form infectious human immunodeficiency virus type 1 pseudotype vector particles, but fail to incorporate upon substitution of the cytoplasmic domain with that of Gibbon ape leukemia virus

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The wild-type (wt) envelope (Env) proteins of spleen necrosis virus (SNV), together with the transmembrane (TM) protein fused to antibody domains (scFv), have been used for the generation of stable packaging cell lines releasing pseudotyped cell targeting vectors derived from SNV and Murine leukemia virus (MLV). As a first step towards assessing whether HIV-1(SNV/TM-scFv) packaging cells could be established for the production of lentiviral cell targeting vectors, it is reported here that infectious HIV-1-derived particles pseudotyped with wt SNV Env proteins could be generated. Using novel chimeric SNV-derived Env proteins encompassing wt and engineered cytoplasmic domains (C-tail) of the Gibbon ape leukemia virus (GaLV) TM protein, it was further shown that the wt C-tail not only excludes the GaLV TM protein from incorporation into HIV-1 particles, but confers this phenotype to other retroviral envelopes upon C-terminal fusion.

Lentiviral vectors, including those derived from Human immunodeficiency virus type 1 (HIV-1), allow efficient gene transfer into non-proliferating cells and are consequently favourable over onco-retroviral vectors for applications in gene therapy (Naldini et al., 1996; Miyoshi et al., 1999). To overcome the narrow CD4-dependent tropism mediated by wild-type (wt) HIV-1 envelope (Env) proteins, HIV-1 particles have been pseudotyped with heterologous Env proteins derived from a variety of parental viruses including Murine leukemia virus (MLV), the G protein of Vesicular stomatitis virus (VSV-G), engineered variants of Gibbon ape leukemia virus (GaLV) and others. This has enabled the transfer of genes into a broad range of different cell-types (Naldini et al., 1996; Stitz et al., 2000; Leurs et al., 2003; Muhlebach et al., 2003; Relander et al., 2005; Merten et al., 2005). Moreover, cell targeting vector particles are desirable at facilitating selective gene delivery into specific cell-types. Among other approaches, the avian spleen necrosis virus (SNV) wt Env proteins, together with hybrid SNV transmembrane (TM) Env proteins N-terminally fused to antibody domains (scFv), have been successfully used to generate stable SNV- and MLV-based pseudotype packaging cells capable of releasing highly cell-type-specific vectors. The high specificity to target cells is due to scFv-mediated binding to a cell-type-specific cell surface protein. It is hypothesized that the scFv anchors the vector particle to the cell surface and thereby enables interaction of the wt SNV Env protein with a mutated human receptor – otherwise not facilitating cell entry and the consequent membrane fusion (Jiang et al., 1998). This strategy of cell targeting has to date only been used for the generation of onco-retroviral particles (Jiang et al., 1998; Engelstadter et al., 2000, 2001). To assess the feasibility of establishing stable HIV-1-derived packaging cells, we attempted to generate infectious HIV-1 vector particles pseudotyped only with the wt Env proteins of SNV as a first step for the future production of lentiviral cell targeting vectors.

The GaLV Env proteins, like the SNV and other retroviral Env proteins, consists of a surface Env protein, which determines cell surface receptor recognition, and a TM protein that regulates incorporation and fusogenicity of the Env proteins. A known determinant for fusogenicity is the so-called R peptide within the C-terminal cytoplasmic domain (C-tail) of the TM protein. The R peptide must be cleaved by a viral protease during virion maturation to mediate virus cell entry (Green et al., 1981; Henderson et al., 1984; Engelstadter et al., 2001; Bobkova et al., 2002). However, the R peptide, or more specifically the protease cleavage site of the C-tail of GaLV Env, has been shown to inhibit incorporation of GaLV TM proteins into HIV-1-derived particles, but confers this phenotype to other retroviral envelopes upon C-terminal fusion.
particles (Christodouloupolous & Cannon, 2001). This inhibitory effect has been overcome by (i) the substitution of the GaLV Env protein C-tail with that of the amphotropic MLV (MLVa) TM protein encompassing its own R peptide and protease cleavage site that is known to enable Env activation by the protease of HIV-1 (Stitz et al., 2000), (ii) the deletion of the R peptide in an engineered GaLV Env protein, or (iii) the modification of the protease cleavage site preceding the R peptide in the GaLV Env C-tail to restore cleavage (Christodouloupolous & Cannon, 2001). The mechanism underlying the exclusion of wt GaLV Env protein from incorporation into HIV-1 is unknown. In addition, it is not yet known whether the C-tail of the wt GaLV TM protein mediates exclusion from HIV-1 incorporation when fused to other retroviral or cellular TM proteins, which are ordinarily incorporated in their wt form. To investigate this, and to possibly enhance HIV-1(SNV) pseudotype vector production, we generated chimeric SNV Env variants harbouring wt and engineered cytoplasmic domains of the GaLV TM protein in place of the SNV TM C-tail, and tested their cell surface expression, incorporation into HIV-1 vector particles and their capability to mediate formation of infectious HIV-1 vector particles in comparison to wt SNV Env.

DNA fragments encoding SNV-derived Env protein variants comprising the wt or engineered C-tails of the GaLV TM protein were generated using PCR and inserted into the expression construct ALF (Stitz et al., 2000; oligonucleotide sequences and a detailed description of the cloning strategy are available upon request). Plasmid pALF-MLVa-wt encoding the wt Env protein of amphotropic MLV has been described earlier (Stitz et al., 2000). In Fig. 1, the predicted amino acid sequences of the C-tails of the engineered chimeric SNV Env variants are compared to those of wt GaLV Env (GaLV-wt), wt amphotropic MLV Env (MLVa-wt) and wt SNV Env proteins (SNV-wt; Bobkova et al., 2002). The Env protein variant SNV-CGawt comprises an SNV Env protein which, in place of the SNV TM protein C-tail, contains the corresponding wt GaLV TM protein C-tail including the predicted protease cleavage site (ILVL) and the R peptide (VLRQKYQALENEGNL). In variant SNV-CGaprtM, 2 aa substitutions have been made from SNV-CGawt, thus introducing the amino acid sequence of the R peptide cleavage site of MLV (QALVL). In construct SNV-CGαAR, the R peptide coding region has been deleted from the GaLV Env C-tail.

We initially examined the ability of the Env protein variants depicted in Fig. 1 to mediate formation of infectious MLV and HIV-1 pseudotype particles. To generate MLV-derived vector particles transferring the reporter gene lacZ, 293T cells were transiently co-transfected with one of the variant Env protein-encoding plasmids, packaging signal-positive MLV-derived transfer vector HIT111 encoding β-galactosidase and packaging construct HIT60 comprising MLV gag–pol (Soneoka et al., 1995). To generate HIV-1-derived vector particles, HIV-1 transfer vector HR’CMVlanZ and packaging construct CMVΔ8.2 (Naldini et al., 1996) were co-transfected together with one of the Env protein-encoding constructs. Cell-free supernatants of the packaging cells were harvested 2 days post-transfection and subjected to titration in canine D17 osteosarcoma cells (susceptible to SNV-infection), murine NIH-3T3 fibroblasts and human HT1080 fibrosarcoma cells (both non-permissive) using the X-Gal-assay as described elsewhere (Stitz et al., 2000). Supernatants from env-negative packaging cells served as negative controls and failed to transduce target cells. Importantly, all onco-retroviral and lentiviral vectors pseudotyped with the wt SNV Env protein, but not with MLVa-wt Env, were unable to transduce murine NIH-3T3 and human HT1080 cells, but mediated gene transfer into canine D17 cells, thus reflecting the host cell tropism of the parental SNV Env protein (data not shown). As shown in Fig. 2(a), all Env protein variants facilitated formation of infectious MLV vector particles with titres from $8\times10^{6}$ infectious units ml$^{-1}$ (i.u. ml$^{-1}$; SNV-CGawt) to a maximum of up to $6\times10^{7}$ and $5\times10^{6}$ i.u. ml$^{-1}$ (SNV-CGαAR and SNV-wt, respectively) proving the functionality of the constructed Env variants. Pseudotyping of HIV-1 vectors with the wt Env proteins of SNV and of amphotropic MLV yielded titres one magnitude lower than those of the MLV vectors displaying the respective identical wt Env proteins. This nevertheless clearly demonstrated the formation of infectious HIV-1(SNV) and HIV-1(MLV) pseudotype vector particles. Consistently with the results obtained for MLV vectors, HIV-1 particles pseudotyped with the R peptide-deleted Env SNV-CGαAR yielded the highest titres ($1\times10^{5}$ and $2\times10^{5}$ i.u. ml$^{-1}$) among all engineered SNV Env variants, even exceeding HIV-1(MLVa-wt) and...
HIV-1(SNV-wt) vector infectivity. Interestingly, the chimeric SNV-CGawt Env protein failed to mediate infectious HIV-1 particle formation. This obviously resulted from the substitution of the SNV TM protein C-tail with that of the wt GaLV TM protein. However, when the presumed protease cleavage site was modified to restore the homologue MLV Env region (SNV-CGaprtM), infectious HIV-1 vector particles were formed. As the vector titres obtained with HIV-1 vectors pseudotyped with the wt Env proteins of MLV and SNV were significantly lower than those of the respective MLV pseudotype vectors, we assumed that the comparatively high titres obtained using Env variants SNV-CGaprtM and SNV-CGaAR resulted from more efficient R peptide processing or more efficient incorporation into lentiviral particles, or both.

Next, we performed Western blot analysis of HIV-1 pseudotype vectors to examine Env protein incorporation and R peptide cleavage. 293T cells were co-transfected with HIV-1-derived packaging and transfer vector constructs and respective Env protein encoding plasmids to generate HIV-1 pseudotype vectors. Untransfected cells and cells transfected only with the transfer vector and packaging constructs served as negative controls. Cell-free supernatants harvested 2 days post-transfection were pelleted by ultracentrifugation through a 20% sucrose cushion and subjected to Western blot analysis on a 10% SDS-PAGE employing anti-SNV-TM (11B11A; Cui et al., 1986) and anti-HIV-1 Gag p24/p55-CA-specific antibodies (MAB880-A; Chemicon) to ensure that similar amounts of vector particles were analysed. Detection of viral proteins was performed using anti-mouse immunoglobulin G1 (IgG1)-horseradish peroxidase-conjugated antibodies and the Enhanced Chemiluminescence Plus detection reagent (both Amersham). As expected, the SNV-wt Env protein was incorporated and partially processed resulting in the formation of infectious HIV-1(SNV) pseudotype vector particles (Fig. 2a) as revealed by the detection of TM proteins with an apparent molecular mass of 16 and 12 kDa (Fig. 2b). The SNV-CGaprtM Env protein was also partially processed by the protease of HIV-1, but was more efficiently incorporated as indicated by the strong TM protein bands of 16 and 12 kDa. The R peptide deleted variant, SNV-CGaAR, exhibited even greater incorporation efficiency as shown by bands of high intensity corresponding to an apparent molecular mass of 12 kDa. These findings could explain the comparatively high HIV-1 pseudotype vector titres obtained using Env variant SNV-CGaAR. Interestingly, the C-tail of the wt GaLV TM protein mediated exclusion of the SNV Env protein from incorporation into HIV-1 vectors (construct SNV-CGawt). Thus, formation of infectious vector particles was not detected (Fig. 2a). Only the unprocessed forms of all TM protein variants were detected in lysates of transfected cells (data not shown), indicating that the cleavage of the R peptide was mediated by the HIV-1 protease during vector particle maturation.

To investigate whether the differential titres of HIV-1 pseudotype vectors and Env protein incorporation were the result of different levels of cell surface expression of the variant Env proteins, 293T cells were transiently co-transfected with one of the various Env constructs, the HIV-1 packaging construct and an EGFP-encoding plasmid, which was used to confirm comparable transfection efficiencies (data not shown). Two days post-transfection, the cells were subjected to fluorescence-activated cell-sorting (FACS) analysis using the SNV TM protein-specific antibody 11A25 (Cui et al., 1986) and anti-mouse-IgG1 antibodies coupled with phycoerythrin (PE; Sigma). As illustrated in Fig. 3, all Env protein variants were displayed on the cell surface of transfected cells at a similar percentage of between 15-9% and 21-5%. Thus, the observed differential incorporation efficiencies and pseudotype vector titres did not result from different cell surface expression levels of SNV-based Env proteins.

In this paper, we have demonstrated for the first time the generation of HIV-1 pseudotype vector particles using the
wt Env proteins of SNV. The unprocessed and the R peptide-deleted form of the SNV TM protein were shown to be incorporated into HIV-1 particles and infectious HIV-1-(SNV) pseudotype vectors were formed, which were capable of efficient transduction of canine D17, but not murine NIH-3T3 and human HT1080 cells, thus exhibiting the typical tropism of SNV. These findings establish a means for the generation of stable HIV-1-based packaging cells expressing the wt SNV Env proteins for the production of high-titre HIV-1(SNV) vectors. The co-expression of SNV TM proteins fused to cell-type-specific scFv may enable the preparation of highly specific targeting vectors, which will be valuable for gene therapy purposes as previously shown for MLV(SNV-wt/SNV TM-scFv) pseudotype vectors (Engelstader et al., 2001).

Chimeric SNV-based Env proteins were constructed to study the influence of wt and engineered C-tails of the GaLV Env protein on the incorporation of heterologous membrane proteins into HIV-1 particles. Collating the results of the titration, the Western blot analysis of the HIV-1 pseudotype particles and the analysis of the cell surface expression of the respective Env proteins, a hypothesis concerning the functionality of the GaLV TM protein C-tail can be described. The SNV-CGaprtM Env protein mediates formation of infectious HIV-1 vector particles as a result of its cell surface expression, presumed co-localization with HIV-1 Gag proteins during virion assembly and budding, and subsequent R peptide cleavage by the HIV-1 protease. This is due to its MLV TM protein-derived protease cleavage site, which allows cleavage by the HIV-1 protease (Kiernan & Freed, 1998; Stitz et al., 2000; Christodouloupolos & Cannon, 2001) and consistent with earlier reports describing viral protease-mediated cleavage of other Env proteins of C-type HIV-1 vector particles as a result of its cell surface expression, presumed co-localization with HIV-1 Gag proteins during virion assembly and budding, and subsequent R peptide cleavage by the HIV-1 protease. This is due to its MLV TM protein-derived protease cleavage site, which allows cleavage by the HIV-1 protease (Kiernan & Freed, 1998; Stitz et al., 2000; Christodouloupolos & Cannon, 2001) and consistent with earlier reports describing viral protease-mediated cleavage of other Env proteins of C-type onco-retroviruses during virion maturation including MLV (Green et al., 1981; Henderson et al., 1984; Rein et al., 1994; Zhao et al., 1998; Bobkova et al., 2002). In contrast, Env protein variant SNV-CGawt harbouring the wt C-tail of the GaLV Env protein, and thus differing in only 2 aa residues from the SNV-CGaprtM protein is excluded from incorporation into HIV-1 vector particles in spite of its comparable cell surface expression and its functionality as demonstrated by its ability to form infectious MLV pseudotype vectors. We therefore believe that the co-localization of the chimeric SNV-CGawt Env proteins with the HIV-1 Gag proteins may have been inhibited by the GaLV Env C-tail, thus preventing incorporation for the GaLV Env proteins, as previously described (Christodouloupolos & Cannon, 2001). Consistent with this notion, Sandrin et al. (2004) recently reported that the lack of intracellular localization and interaction between feline endogenous retrovirus RD114 Env and simian immunodeficiency Gag proteins restricted the formation of pseudotype particles resulting from specific signal sequences in the C-tail of RD114 Env. As the exchange of only 2 aa in the cytoplasmic domain of the TM protein of Env variant SNV-CGaprtM was sufficient to overcome the block of incorporation, we conclude that this inhibition of incorporation resulted from sequence motifs located in the proximity of the presumed protease cleavage site. This is further supported by the findings of Christodouloupolos & Cannon (2001), who demonstrated that alteration of the assumed protease cleavage site is sufficient to restore incorporation of GaLV Env proteins. Moreover, the data published by Merten et al. (2005) argue for a dual function, namely protease substrate and localization signal, of the amino acid residues surrounding the R peptide cleavage site in the C-tail of the wt GaLV Env protein. However, in this report, heterologous viral TM proteins comprising the cytoplasmic domain of the wt GaLV Env protein were clearly excluded from incorporation into HIV-1 particles. The transferability of this characteristic phenotype to other proteins strongly suggests that the C-tail harbours a signal not restricted to the N-terminal portions of its parental GaLV TM protein. Whether this phenotype can be transferred to cellular or other viral TM proteins upon fusion to the wt GaLV Env C-tail remains unknown. Further research aimed at deciphering the co-localization signals of further viral proteins during assembly and budding will be necessary to gain a deeper understanding of the fundamental mechanisms facilitating or restricting efficient lentivirus pseudotype formation.

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References


