Truncation of the enzootic nasal tumor virus envelope protein cytoplasmic tail increases Env-mediated fusion and infectivity

Scott R. Walsh, Jondavid G. de Jong, Jacob P. van Vloten, María Carla Rosales Gerpe, Lisa A. Santry and Sarah K. Wootton*

Abstract

Enzootic nasal tumor virus (ENTV) and Jaagsiekte sheep retrovirus (JSRV) are highly related ovine betaretroviruses that induce nasal and lung tumours in small ruminants, respectively. While the ENTV and JSRV envelope (Env) glycoproteins mediate virus entry using the same cellular receptor, the glycosylphosphatidylinositol-linked protein hyaluronoglucosaminidase, ENTV Env pseudovirions mediate entry into cells from a much more restricted range of species than do JSRV Env pseudovirions. Unlike JSRV Env, ENTV Env does not induce cell fusion at pH 5.0 or above, but rather requires a much lower pH (4.0–4.5) for fusion to occur. The cytoplasmic tail of retroviral envelope proteins is a key modulator of envelope-mediated fusion and pseudotype efficiency, especially in the context of virions composed of heterologous Gag proteins. Here we report that progressive truncation of the ENTV Env cytoplasmic tail improves transduction efficiency of pseudotyped retroviral vectors and that complete truncation of the ENTV Env cytoplasmic tail increases transduction efficiency to wild-type JSRV Env levels by increasing fusogenicity without affecting sensitivity to inhibition by lysosomotropic agents, subcellular localization or efficiency of inclusion into virions. Truncation of the cytoplasmic domain of ENTV Env resulted in a significant advantage in viral entry into all cell types tested, including foetal ovine lung and nasal cells. Taken together, we demonstrate that the cytoplasmic tail modulates the fusion activity of the ENTV Env protein and that truncation of this region enhances Eenv-mediated entry into target cells.

INTRODUCTION

Enzootic nasal tumor virus (ENTV) and Jaagsiekte sheep retrovirus (JSRV) are simple betaretroviruses of small ruminants that induce tumours in the nose [1] and lung [2], respectively. Despite sharing approximately 89 % nucleotide identity [3], JSRV and ENTV have distinctly different tissue tropisms. While the long terminal repeat (LTR) of JSRV is preferentially active in type 2 alveolar cells [4], the LTR of both ENTV and JSRV has been shown to be active in the nose, trachea and lung epithelium of mice [5], suggesting that the LTR may not be the only component of the virus responsible for disease tropism. The envelope glycoprotein (Env) of ENTV and JSRV is a potent oncogene, and expression of Env in vivo has been shown to induce tumours [6, 7], but not in a tissue-specific manner [5, 8]. The cellular receptor for ENTV-1 and JSRV is hyaluronoglucosaminidase 2 (Hyal2) [9–11], a glycosylphosphatidylinositol-anchored protein and a member of a group of proteins that degrade hyaluronan [12, 13]. While Hyal2 appears to be the primary receptor for both viruses, JSRV-Env-pseudotyped retroviral vectors can transduce cells from a wide range of species including human, sheep, monkey, dog, cow and rabbit, whereas ENTV Env mediates entry into cells from a much more restricted range of species and with much lower efficiency [14, 15].

For many retroviruses, the cytoplasmic tail domain of Env modulates a variety of functions including cell surface expression, stability, fusogenicity, efficiency of virion incorporation and infectious potential [16–19]. JSRV Env (Jenv) and ENTV-1 Env (Eenv) share very high amino acid identity (~93 %) across most of their protein coding sequence with the exception of their cytoplasmic tails, which share only 50 % amino acid identity. Whereas Jenv possesses one tyrosine-based (Yxxθ, where θ represents a
hydrophobic amino acid) sorting motif in its cytoplasmic tail, Eenv contains two tyrosine as well as two dileucine (LD) sorting motifs [20]. These motifs are found in the cytoplasmic tail of many viral glycoproteins, including Moloney murine leukemia virus (MoMLV) and Mason–Pfizer monkey virus, and have been shown to influence subcellular localization and pseudotyping efficiency [21–23].

To better understand why Eenv mediates infection so inefficiently, particularly in comparison to its close relative, Jenv, we progressively truncated and/or mutated the cytoplasmic tail of Eenv and examined the effect this had on surface expression, virion incorporation, fusogenicity and ability to facilitate entry into target cells in vitro. Partial truncation resulting in removal of all tyrosine-based motifs did not significantly affect transduction efficiency or infectivity of Eenv pseudovirions; however, complete truncation of the cytoplasmic tail (Eenv I575*) dramatically increased transduction efficiency by increasing fusion at pH 5. Mutation of the dileucine motifs in the cytoplasmic tail of Eenv mutants lacking tyrosine-based motifs did not significantly alter subcellular localization; however, transduction efficiency and fusion were increased to a level near that of the fully truncated Eenv protein. Lentivirus vectors pseudotyped with either Jenv or Eenv I575* showed similar infectivity in vitro in primary foetal lung and nasal cells, suggesting that removing the cytoplasmic tail of Eenv dramatically improves infectivity of vectors dependent on Eenv to mediate entry into cells.

**RESULTS**

**Truncation of the cytoplasmic tail of Eenv enhances transduction efficiency of pseudotyped virions**

To examine the role of the cytoplasmic tail of Eenv in retroviral transduction, site-directed mutagenesis was used to modify or truncate the cytoplasmic tail of Eenv at the amino acid positions indicated in Fig. 1a. NIH 3T3/LI2SN cells engineered to express human Hyal2 [10] were transduced with MoMLV or human immunodeficiency virus (HIV) vectors pseudotyped with the various Eenv mutants, and the number of alkaline phosphatase (AP)-positive foci were compared to that of cells transduced with wild-type Jenv- and Eenv-pseudotyped viruses. Results were reported as a percentage of the number of AP-positive foci in cells transduced with pseudotyped MoMLV (white bar) or HIV (grey bar) virions, with Jenv set at 100 % (Fig. 1b). The transduction efficiency of Eenv pseudovirions was significantly lower than that of Jenv pseudovirions; however, complete truncation of the Eenv cytoplasmic tail (represented by the Eenv I575* mutant) dramatically increased transduction efficiency, approaching that of Jenv (Fig. 1b). Similarly, removing the cytoplasmic tail of Eenv increased transduction efficiency of pseudotyped virions in primary cells of ovine origin (Fig. S1, available in the online Supplementary Material). The truncation mutant lacking the previously described transforming tyrosine-based motif (EenvY590*, Fig. 1a) was not significantly different from wild-type Eenv (Fig. 1b), nor were truncation mutants D601* or S609* (data not shown). Since the Y590* truncation mutant did not increase transduction efficiency, we reasoned that an element within the intervening sequence (amino acids 575–590) must be inhibiting transduction. Analysis of the cytoplasmic tail sequence of Y590* revealed the presence of two dileucine motifs (LI residues shown in red in Fig. 1a), which are known to play a role in internalization and trafficking [20, 24], that are conserved in Eenv, but absent from Jenv. The second hydrophobic residue of each of the dileucine motifs was mutated to serine (I575S and I586S), in order to ablate the motif and any associated putative trafficking function [22]. Dileucine mutations were introduced in the context of the Y590* truncation (diLY590*), and a dramatic increase in transduction efficiency for both vector systems was observed. The diLY590* mutant displayed transduction efficiencies comparable to that of the I575* mutant, suggesting that the dileucine residues in this region of the Eenv protein impede vector pseudotyping or infection. These results demonstrate that the amino acid sequence proximal to the membrane-spanning domain (MSD), particularly the two dileucine motifs, inhibits the transduction efficiency of Eenv-pseudotyped viruses.

**Cytoplasmic tail modification of Eenv does not affect co-localization with Gag**

Confocal microscopy was employed to determine whether truncation of the cytoplasmic tail or mutation of the membrane-proximal dileucine motifs alters co-localization of Eenv with MoMLV or HIV Gag. MoMLV Gag–YFP and HIV Gag–GFP fusion proteins were co-expressed with the various Eenv expression constructs in HEK 293 cells, and envelope proteins were immunolabelled with a murine polyclonal anti-Env primary antibody followed by Texasred-conjugated anti-mouse secondary antibody. Co-localization of the different ENTV-1 envelope proteins with either MoMLV (Fig. 2) or HIV (data not shown) Gag did not differ substantially between wild-type and mutant envelopes, with the exception of the Y590* mutant, which appeared to accumulate to a greater extent within an intracellular compartment, with only a couple of cells showing plasma membrane localization. Both MoMLV and HIV Gag localized with Env in distinct punctate areas of fluorescence. The majority of YFP/GFP punctate structures were closely associated with a more diffuse cluster of fluorescence in the red channel, which was demonstrated as a yellow area with a red halo in the merged image. Interestingly, these foci of co-localization were observed in intracellularly as well as at the plasma membrane. Results from these experiments suggest that complete truncation of the Eenv cytoplasmic tail does not dramatically affect co-localization with either MoMLV or HIV Gag, but that removal of the terminal 28 amino acids, which contains the YxxM motif, leads to greater intracellular accumulation.
Truncation or modification of the Eenv cytoplasmic tail does not affect surface expression but can influence envelope glycoprotein incorporation into virions

Since MoMLV and HIV particles assemble and bud from the plasma membrane, localization of the envelope protein on the cell surface is necessary for inclusion into virions.

To address whether modification of the Eenv cytoplasmic tail altered cell surface expression, the relative level of envelope protein surface expression in transiently transfected HEK 293 cells was quantified by flow cytometry using murine anti-Env polyclonal antiserum. A shift in fluorescence intensity relative to the 10A1 control envelope was observed in all cell populations expressing wild-type Eenv.

**Fig. 1.** Truncation of the Eenv cytoplasmic tail increases transduction efficiency. (a) Schematic of truncations and mutations introduced into the cytoplasmic tail of Eenv. The cytoplasmic tail spans amino acid positions 572–617 of the ENTV envelope protein. Tyrosine-based motifs [81] and putative dileucine motifs in the cytoplasmic tail sequence are shown in blue and red, respectively. Bolded black letters indicate amino acids that either do not constitute or no longer constitute a dileucine motif. Truncation mutant constructs were named according to the amino acid at which the truncating stop codon was introduced. diL indicates constructs in which the two membrane-proximal dileucine motifs have been abolished by mutation of the isoleucines at amino acid position 575 and 586 to serine. SU, surface subunit; ED, ectodomain; TM, transmembrane subunit. (b) NIH 3T3 cells overexpressing human Hyal2 (NIH 3T3/LL2SN) were transduced with MoMLV or HIV virions pseudotyped with the indicated Env protein, and the number of transduced foci was counted 48 h later following staining for alkaline phosphatase marker gene expression. Foci counts are expressed as a percentage of Jenv-pseudotyped MoMLV or HIV virion transduction from five independent experiments with Jenv set to 100%. Mock refers to cells treated with supernatant from mock-transfected cells. The average transduction by virus bearing Jenv was $5 \times 10^4$ transducing units ml$^{-1}$. Two-way ANOVA was used for statistical analysis, and error bars indicate standard error of the mean. The lines above the bar graph denote the accompanying statistical difference for the different envelopes in relation to Eenv. The $P$-value directly above each line is associated with the difference between only the outermost two groups of bars underneath the line. NS, Not significant.
type and mutant Env proteins (Fig. 3a) as was a similar mean fluorescence intensity (Fig. 3b), indicating that modification of the cytoplasmic tail had little effect on surface expression of Eenv.

To test the hypothesis that differences in transduction efficiency between wild-type Eenv and cytoplasmic-tail-truncated Eenv proteins could be due to differences in the efficiency of Env glycoprotein incorporation, envelope inclusion into MoMLV- and HIV-based vectors was investigated by analysing Env-SU [surface subunit (SU) domain]-to-capsid (Ca) protein ratios of equal volumes of concentrated pseudotyped virus particles by Western blot analysis. A single Western blot representative of four independent experiments is shown for MoMLV (Fig. 3c, top) and HIV (Fig. 3c, bottom) vector systems. Note that, in our hands, the SU domain of Eenv routinely migrates at approximately 63 kDa, whereas the SU domain of Jenv typically migrates at 60 kDa. The ratio of Env-SU to capsid protein for each experiment was determined using densitometry and the average values graphed (Fig. 3d). Lower values suggest less efficient Env incorporation. Wild-type Eenv and the cytoplasmic tail mutants, Eenv I575*, Eenv Y590* and Eenv diLY590*, all had higher Env-SU : capsid ratios in MoMLV virions compared to Jenv. However, the Env-SU : capsid ratio of Eenv I575* in MoMLV pseudovirions was significantly lower than that of the other Eenv proteins. This trend was not observed in the HIV vector system. In this case, Jenv, Eenv I575* and Eenv Y590* pseudovirions all had similar Env-SU : capsid ratios that were significantly higher than Eenv and Eenv diLY590*. Taken together, these results indicate that truncation of the cytoplasmic tail of Eenv does not significantly interfere with cell surface expression and that

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**Fig. 2.** Co-localization of MoMLV Gag with ovine betaretrovirus envelope proteins. HEK 293 cells were co-transfected with the indicated pClneoJenv, Eenv or Eenv mutant constructs and MoMLVgagYFP expression vector for 48 h before fixation and permeabilization. Polyclonal mouse antibody raised against the JSRV Env protein (that cross-reacts with the ENTV Env protein) was detected with a Texas-red-conjugated goat anti-mouse secondary antibody. Nuclei were stained with DAPI. Representative confocal microscopy images are shown.
Fig. 3. Effect of Eenv cytoplasmic tail truncation on cell surface expression and envelope glycoprotein incorporation into virions. (a) HEK 293 cells transiently transfected with expression vectors for the indicated ovine betaretrovirus Env proteins were stained with polyclonal mouse antibody raised against ovine betaretrovirus Env proteins and Alexa-Fluor-488-labelled anti-mouse secondary antibody and analysed by flow cytometry. Dead cells and cells with compromised membrane structures were excluded based on PI staining. Coloured lines represent the fluorescence shifts of the corresponding ovine betaretrovirus Env proteins relative to the grey dotted line, which represents cells transfected with an irrelevant Env from 10A1MLV. (b) Mean fluorescence intensity (MFI) of Env expression. (c) Western blot images representative of four independent experiments showing Env protein (SU domain only) inclusion in MoMLV (top) and HIV (bottom) pseudotyped virions relative to capsid (Ca) protein. (d) Band intensities from four separate experiments were determined by densitometry, and the average ratio of Env-SU to capsid protein density is graphed. Data are presented as mean±SEM. Two-way ANOVA was performed followed by Tukey’s multiple comparison test. Statistically significant differences (P<0.05) between the Env-SU: capsid protein ratios are indicated by different letters.
modification of the cytoplasmic tail can influence the efficiency of Env incorporation into MoMLV and HIV virions, but this does not correlate with differences in transduction efficiency.

**Lysosomotropic agent and V-ATPase inhibitor treatments have similar effects on wild-type and cytoplasmic-tail-truncated Eenv pseudovirion transduction**

Fusion by Jenv- and Eenv-pseudotyped virions is inhibited by lysosomotropic agents/V-ATPase inhibitors and is increased at low pH, indicating that fusion takes place in the late endosome/early lysosomal compartments [25, 26]. To investigate whether this low pH requirement was maintained in the Eenv truncation mutants, we generated MoMLV viruses pseudotyped with Jenv, Eenv, Eenv I575*, Eenv Y590* and Eenv diLY590* and tested their abilities to enter NIH 3T3/LL2SN cells in the presence of bafilomycin A1, a highly selective nonreversible endosomal proton pump (V-ATPase) inhibitor [27], as well as two commonly used lysosomotropic agents: ammonium chloride (NH4Cl) and chloroquine [28]. Pseudovirions bearing the envelope glycoproteins of vesicular stomatitis virus (VSV)-G or 10A1 were included as positive and negative controls, respectively. As expected, entry by pH-dependent VSV-G-pseudotyped viruses was nearly abolished by treatment with bafilomycin A1 or lysosomotropic agents [29], while 10A1 Env-pseudotyped viruses were not affected by treatment with these agents (Fig. 4), consistent with the fact that 10A1 is a pH-independent retrovirus [30, 31]. A significant difference between untreated and all treatments was observed in cells transduced with Jenv-pseudotyped viruses, as was observed previously [25]. Transduction by Eenv-pseudotyped viruses was significantly inhibited by NH4Cl and bafilomycin A1 treatment; however, even though fewer AP-positive foci were observed in chloroquine-treated wells compared to untreated wells, this reduction was not statistically significant (Fig. 4). The same pattern of inhibition was observed for Eenv-Y590* and Eenv diLY590*-transduced wells. Altogether, these results suggest that truncation of the cytoplasmic tail does not affect the pH-dependent entry of Eenv-pseudotyped virions.

**Fusogenicity of Eenv is increased by cytoplasmic tail truncation and dileucine motif mutation**

The ability of a viral envelope protein to mediate fusion is essential for infection to occur. Thus, we investigated the fusogenic properties of the Eenv cytoplasmic tail truncation mutant relative to wild-type envelopes. For this experiment, HEK 293T cells co-expressing Tat and Env [32, 33] were co-cultured with MAGI cells engineered to express human Hyal2 and after 24 h, treated with pH 5 citrate buffer for 5 min. nLacZ-positive syncytia were identified after Xgal staining, and only syncytia containing three or more nuclei were counted. Highly fusogenic VSV-G protein was included as a positive control and, as expected, produced numerous syncytia (Fig. 5a) containing 10 or more nuclei (~700 foci of fused cells/well, Fig. 5b). Foci of fused cells were rarely detected in Jenv (~21 foci of fused cells/well, Fig. 5b) and wild-type Eenv-transfected cells (~7 foci of fused cells/well, Fig. 5a and b) and were often composed of only three to five nuclei. Conversely, syncytia in Eenv I575* (Fig. 5a) and Eenv diLY590*-transfected cells contained up to seven or eight nuclei and approximately 235–148 foci of fused cells/well, respectively (Fig. 5b). Data were analysed using two-way ANOVA followed by Tukey’s multiple comparison test and statistical differences were found between Jenv/Eenv and Eenv I575* (P < 0.001) as well as Jenv/Eenv and Eenv diLY590* (P < 0.05) (Fig. 5b). There was no significant difference between Eenv I575* and Eenv diLY590*. These results suggest that truncation of the Eenv cytoplasmic tail serves to enhance the fusogenicity of this protein.

**The sequence proximal to the MSD of Eenv does not promote association with detergent-resistant membranes**

The region of the cytoplasmic tail immediately proximal to the MSD of Jenv was previously shown to form an
amphipathic helix structure that when fused to GFP altered its localization, resulting in a punctate pattern of fluorescence throughout the cytoplasm and exclusion from the nucleus [34]. Furthermore, removal of the amphipathic helix from Jenv was found to increase its fusogenicity [35]. Since removal of the same region proximal to the MSD in the cytoplasmic tail of Eenv increases fusogenicity, it is possible that Eenv might encode an amphipathic helix that, like Jenv, promotes membrane association and modulates fusion activity. To address this possibility, the first 18 amino acids of the cytoplasmic tail of Eenv and Jenv were fused to the amino terminus of Clover GFP (Fig. 6a), a mutant version of GFP with greater dynamic range and photostability [36]. To test whether fusing the putative amphipathic helix of Jenv and Eenv to Clover would increase association with cellular membranes, a membrane floatation assay was conducted. Briefly, HEK 293T cells transfected with Jenv Amphi, Eenv Amphi or Clover were lysed in 1 % Triton X-100 and subjected to 5–30 % discontinuous sucrose gradient ultracentrifugation. Fractions were collected and analysed by Western blot analysis for Clover expression (Fig. 6b). Using this approach, membranes and membrane-associated proteins float up to reach equilibrium in the top fractions of the gradient, whereas cytosolic proteins remain at their original loading position in the bottom fractions of the gradient [37]. Fusion of the JSRV amphipathic helix resulted in a shift of Clover to fraction 2 (lower density), suggesting increased association with membranes, whereas fusion of the putative amphipathic helix from Eenv did not alter the membrane association of Clover (Clover remained primarily at the densest part of the gradient in fractions 5 and 6). Taken together, these results suggest that, unlike Jenv, the
membrane-proximal cytoplasmic tail domain of Eenv does not enhance association with membranes.

**DISCUSSION**

The objective of this study was to investigate the role of the cytoplasmic tail of ENTv Env in modulating virus infectivity. To this end, we employed a strategy whereby we progressively truncated and/or mutated the cytoplasmic tail of Eenv and evaluated these mutants for surface expression, virion incorporation, fusogenicity and ability to facilitate entry into target cells. Progressive truncation and/or deletion of dileucine- and tyrosine-based motifs resulted in increased enhancement of Eenv fusogenicity and, consequently, increased viral infectivity. The most dramatic phenotype was observed following complete truncation of the cytoplasmic tail, which suggests that the region immediately following the MSD plays a critical role in modulating Eenv function; however, it does not appear to mediate membrane interactions. Improvement in infectivity correlated with the ability of Eenv to mediate cell-to-cell fusion. Therefore, it is likely that the enhanced transduction efficiency of the fully truncated Eenv-I575*-pseudotyped virions is a result of increased fusogenic activity of Eenv.

There are numerous examples among retroviruses in which mutations in the cytoplasmic tail impact Env function [38–42]. Removal of the cytoplasmic tail domain from retroviral Env proteins [43–48], as well as other viral envelope glycoproteins [49–52], generally increases the infectivity of pseudotyped viruses, suggesting that this region often contains inhibitory sequences. In fact, for some retroviruses, a portion of the cytoplasmic tail is deliberately removed by viral protease cleavage after virion assembly [53]. For example, MoMLV [54–57], gibbon ape leukemia virus [44] and several other retroviral [58, 59] Env proteins contain a short ~16 amino acid sequence at the carboxy terminus of the cytoplasmic tail, termed the R peptide, that regulates fusogenicity of the Env protein. The R peptide needs to be removed to completely activate Env fusogenicity following viral particle release [54, 57, 60–62]. This mode of regulation is thought to be important for viral fitness because it prevents syncytium formation within infected cells but when cleaved from Env during maturation of the virus particle, activates fusion so that newly assembled viruses are competent to infect target cells. If there were inhibitory signals in the cytoplasmic tail of Eenv that prevented fusion, then truncation would eliminate those functions, resulting in increased infectivity of pseudotyped virions. Indeed, we observed that truncation of the Eenv cytoplasmic tail dramatically increased transduction efficiency of both lentivirus and retrovirus pseudotyped particles.
Assembly of both lentiviruses and gammaretroviruses occurs at the plasma membrane, whereas assembly of betaretroviruses, such as Mason–Pfizer monkey virus, occurs intracellularly in association with the microtubule-organizing centre, after which the core is transported to the plasma membrane for envelopment [63]. The mechanisms and interactions required for betaretrovirus assembly are not well understood, but inclusion of betaretroviral envelope glycoproteins into viral vectors composed of heterologous Gag proteins is presumed to occur through passive incorporation of surface-expressed Env proteins [64]. Since all of the mutant Eenv proteins were expressed at the cell surface, including those lacking both dileucine- and tyrosine-based motifs, we can assume that the cytoplasmic tail does not play a critical role in targeting to the plasma membrane. While it is somewhat surprising that deletion of classical endocytic sorting motifs did not alter cell surface expression of Eenv, preliminary results from our lab suggest that these putative endocytic motifs do not influence the rate of Env protein internalization (J. G. de Jong et al., unpublished). To address whether cytoplasmic tail modification altered the efficiency of virion incorporation, we evaluated the ratio of Env-SU to capsid in both MoMLV and HIV virions pseudotyped with the various Eenv mutant proteins. While we observed significant differences in Env-to-capsid ratios for some of the mutants, these differences did not correlate with virus infectivity, nor were they similar between vector systems, suggesting that Env virion incorporation alone is not the major factor regulating infectivity.

The increase in transduction efficiency associated with truncation of the Eenv cytoplasmic tail was not likely due to differences in the dependence on low pH for virus entry since a similar transduction inhibition profile was observed for all of the cytoplasmic tail truncation mutants as well as for wild-type Eenv after treatment with lysosomotropic agents or V-ATPase inhibitors. Since treatment with lysosomotropic agents/V-ATPase inhibitors leads to an increase in the pH of late endosomes and lysosomes, it is difficult to reconcile the inconsistency observed in NH₄Cl/Bafilomycin A1 and chloroquine inhibition of Eenv. Nevertheless, sensitivity to NH₄Cl and V-ATPase inhibitors and resistance to chloroquine treatment have been reported in other viral systems, including equine infectious anemia virus [65], human foamy virus [66] and Sindbis virus [67].

The fusogenic activity of wild-type Eenv and Jenv was relatively inefficient at pH 5 relative to VSV-G; however, removal or disruption of the membrane-proximal structure in the cytoplasmic tail significantly increased the fusogenicity of Eenv at pH 5 and to some extent at pH 7 (data not shown). Therefore, we conclude that truncation of the cytoplasmic tail of Eenv increases the transduction efficiency of Eenv-pseudotyped virus particles by increasing fusion efficiency without significantly affecting localization or virion incorporation. These results suggest that the cytoplasmic tail domain of the Eenv is able to modulate or restrict the fusogenic activity of the Eenv protein, despite the fact that fusion initiates on the extracellular leaflet of the membrane.

It is possible that the Env protein of ENTV evolved to be poorly fusogenic, particularly at neutral pH, as one of its other main functions is to transform cells. Indeed, there are no reports of syncytia formation in tumour cells that have been transformed by high levels of Eenv expression. Therefore, the cytoplasmic tail of Eenv may function to restrict its fusogenic activity to prevent premature activation or syncytia formation in infected cells. The conservation of the cytoplasmic tail sequence among ENTV isolates [3], specifically the additional tyrosine-based motif and the two dileucine motifs, indicates that the cytoplasmic tail is likely to play an important role in the biology and pathogenesis of the virus, factors that cannot be evaluated in the pseudotype system.

Jenv- and Eenv-mediated entry of pseudotyped viruses in primary foetal cells of ovine origin was significantly enhanced compared to murine fibroblasts overexpressing human Hyal2 (NIH 3T3/LL2SN). This could be due to receptor expression levels being higher and more uniform in primary cells of ovine origin as only a proportion of the NIH 3T3-Hyal2 cells express high levels of Hyal2. It is also possible that differences in transduction are due to requirements for supporting host cell factors, such as coreceptors [11, 15], which would more likely be expressed in cells of ovine origin. Indeed, other reports suggest that Hyal2 abundance is not the limiting barrier to Jenv-mediated transduction, at least in well-differentiated human airway epithelia [68].

In summary, removal of the cytoplasmic tail from Eenv dramatically improves the infectivity of pseudotyped lentiviral and gammaretroviral vectors without affecting sensitivity to inhibition by lysosomotropic agents, subcellular localization or efficiency of virion incorporation. Rather, the increased transduction efficiency of Eenv I575* appears to be a consequence of increased fusogenicity.

METHODS
DNA constructs

The vector pSX2.Jenv, containing the LTR and a truncated intron from MoMLV upstream of the JSRV env gene, was obtained from Dr Dusty Miller [69]. The env gene of ENTV-1 was amplified from a plasmid containing a fragment of the ENTV-1NA4 genome (GenBank accession number: FJ744146) using primers to add DraIII (Eenv F) and ClaI (Eenv R) (Table 1) restriction sites to the 5’ and 3’ termini, respectively, and KOD Hot Start DNA Polymerase (Novagen). Both Eenv and Jenv were cloned into the EcoRI site of pCI-neo for expression in mammalian cells after amplification with Eenvsc-F or Jenvsc-F primers, respectively, and the Envsc-R primer (Table 1). Truncation and dileucine mutants shown in Fig. 1 were created according to the modified site-directed mutagenesis protocol described by Wang et al. [70] using the primers listed in Table 1. Amphilpathic helix–Clover fusion (Amphi–Clover) constructs were generated using forward primers harbouring...
putative amphipathic helices (Jenv-Amphi-F and Eenv-Amphi-F) and the Amphi-RV reverse primer (Table 1). PCR amplicons were cloned into pGEM-T Easy according to the manufacturer’s specifications and then subcloned into the MluI and EcoRI sites of pCI-puro.

Cell culture and production of pseudotyped virus

HEK 293, HEK 293T, NIH 3T3 LGPS/LAPSN [71], NIH 3T3/LL2SN [72], HeLa Tat3 and HeLa-CD4-LTR-β-gal (MAGI) [obtained through the National Institutes of Health (NIH) AIDS Reagent Program: HeLa-CD4-LTR-β-gal from Dr Michael Emerman] [73] cells were grown in Dulbecco’s minimal essential medium supplemented with 10 % FBS at 37 °C, 5 % CO2.

For lentivirus production, HEK 293T cells were transfected using the calcium phosphate co-precipitation method with pTYEFnLacZ, pHpDn/A, pCEP4 Tat (obtained through the NIH AIDS Reagent Program), pCMVβ-gal and the appropriate pCI-neo Env expression vectors. VSV-G-pseudotyped viruses were created using the pCMV-VSV-G expression vector (Addgene plasmid 8454) [74]. Pseudotyped MoMLV particles were generated by transfection of NIH 3T3/LGPS-LAPSN cells with pSX2-based envelope expression vectors using the calcium phosphate transfection method. In both cases, medium was replaced after 16 h with 5 ml fresh medium. Supernatants were either used immediately for transduction or stored in aliquots at −80 °C.

Indirect immunofluorescence microscopy

HEK 293 and NIH 3T3/LL2SN cells were seeded onto glass coverslips treated with FNC Coating mix (Anthena) at 1 × 104 cells/well in a 6-well dish 1 day prior to transfection. Envelope constructs were co-transfected with either pHIVGagGFP [obtained through the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH: pGag-EGFP (Cat#11468) from Dr Marilyn Resh] [75] or pMLV-GagYFP (Addgene plasmid #1813) [76]. Cells were fixed and permeabilized 48 h after transfection with 3.7 % formaldehyde-1% TritonX-100-PBS for 10 min at RT. After the cells were washed and blocked, murine hyperimmune serum (1 : 200) directed against the JSRV Env protein was added. Cells were then stained with fluorescence-conjugated secondary antibodies, followed by mounting with Prolong Gold Antifade Reagent (Life technologies) containing DAPI counterstain. Cells were imaged at × 40 with a Leica DM 6000B confocal microscope (Molecular and Cellular Imaging Facility, University of Guelph).

Flow cytometry

HEK 293 cells (2×10⁶ cells) were transiently transfected by the calcium phosphate precipitation method with Env expression vectors. Media was changed 16 h post-transfection, and cells were harvested 48 h post-transfection using PBS/0.2 % EDTA treatment and gentle agitation with a pipette. Buffers were supplemented with 2 % FBS at 4 °C throughout the sample preparation procedure. Cells were treated with PBS-2% FBS before staining with polyclonal mouse serum directed against the JSRV envelope protein (1 : 200). Polyclonal mouse serum was obtained from C57BL/6 mice that had previously been infected with an AAV vector expressing the JSRV Env protein [77]. Cells were washed and stained with an Alexa-Fluor-488-conjugated goat anti-mouse IgG secondary antibody. Finally, cells

Table 1. List of primer sequences utilized in cloning and mutagenesis

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<th>Primer name</th>
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<tr>
<td>Eenv-R</td>
<td>CATCAGTGAGAACGGTGCCTAGTACT</td>
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<td>pSXenvKpnl-F</td>
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<td>pSXenvXbal-R</td>
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<td>Eenv I575S-F</td>
<td>GCCCTACCTGAGATTTAAGCAAAGATTTCCT</td>
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<tr>
<td>Eenv I575S-R</td>
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<td>TATTTCTATGCTGTTAACTACTCTTCTTATTGAG</td>
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<td>Jenv-Amphi-F</td>
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<tr>
<td>Eenv-Amphi-F</td>
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<tr>
<td>Amphi-RV</td>
<td>TAAAGGGTTTTACTTGTCAGGC</td>
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were washed and stained with propidium iodide to exclude non-viable cells and analysed on a FACScan flow cytometer (Becton Dickinson). FlowJo software (Tree Star Inc.) was used to evaluate cell populations gated based on cells stained with secondary antibody alone.

**Immunoblotting**

Pseudotyped virus particles were pelleted by ultracentrifugation through a 20 % sucrose (w/v) cushion in a SW32 Ti rotor (Beckman Coulter Canada) at 60 000 g for 2 h at 4 °C. Pellets were suspended in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 10 mM EDTA, 1 % sodium deoxycholate) supplemented with 1 mM PMSF, 1 mM Na3VO4 and protease inhibitor cocktail (Sigma-Aldrich), resolved on a 12 % SDS-PAGE gel and transferred to PVDF membrane. Membranes were blocked with 5 % skimmed milk in PBS-0.5% Tween 20 and probed with antibodies against the JSRV envelope [78] and the HIV capsid [NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH: Monoclonal Antibody to HIV-1 p24 (AG3.0) from Dr Jonathan Allan] [79] or MoMLV capsid (a kind gift from Dr Stephen Goff, Columbia University).

**Treatment with lysosomotropic agents**

NIH 3T3/LL2SN cells were seeded on 12-well plates (5 x 10^5 cells/well) and, in experiments involving lysosomotropic agents, were pre-treated with 5 mM ammonium chloride or 10 µM chloroquine for 2 h, and infections were performed in the presence of lysosomotropic drugs. No pre-treatment was used for cells treated with bafilomycin A1 (5 nM). NIH 3T3/LL2SN cells were transduced with equivalent amounts of pseudotyped MoMLV or HIV vector containing supernatant as determined using the EnzChek Reverse Transcriptase assay kit (Invitrogen) in the presence of 8 µg ml⁻¹ Polybrene (Sigma) for 16 h. Transduction was quantified 48 h post-infection by fixation of cells with 3.7 % formaldehyde-PBS and staining for AP or nLacZ expression as described previously [11]. Bar graphs were generated from three independent experiments, and statistical analysis was performed using GraphPad. Two-way ANOVA was used to determine statistically significant differences among the different treatment groups transduced with a particular pseudotyped virus. P-values of 0.05 or less were considered to be statistically significant.

**Cell–cell fusion assay**

HEK 293T cells were seeded in 12-well plates at 1 x 10^4 cells/well and transfected with pCEP4Tat and envelope expression vectors by the calcium phosphate method. After 16 h, the media was replaced, and MAGI-Hyal2 cells (MAGI cells engineered to express human Hyal2) were added (1 x 10^4). After 24 h, co-cultures were treated with citrate buffer at pH 5 for 5 min. Treated cells were rinsed twice with PBS and incubated with fresh media for 16 h before fixation with 3.7 % formaldehyde-PBS for 10 min and staining with Xgal staining solution at 37 °C for 16 h. nLacZ-positive syncytia were counted manually. Syncytia containing two or fewer nuclei were excluded.

**Flotation assays**

Flotation assays were conducted as previously described [80]. Briefly, HEK 293T cells transiently transfected with the Amphipathic–Clover fusion constructs were lysed on the dish with soft lysis buffer (1 % Triton X-100, 25 mM Tris HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA) supplemented with protease inhibitors for 30 min at 4 °C. Lysates were harvested and centrifuged at 10 000 g for 5 min at 4 °C. The cleared, postnuclear lysate was adjusted to 40 % (w/v) sucrose, and a 5–30 % discontinuous sucrose gradient was layered on the top. Samples were ultracentrifuged at 100 000 g for 18–24 h at 4 °C. Fractions (500 µl) were collected from the top of the gradient, and 75 µl of each fraction was lysed, resolved by 12 % SDS-PAGE and transferred to PVDF as detailed above before conducting probing with an anti-GFP antibody.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


8. Wootton SK, Halbert CL, Miller AD. Envelope proteins of jaagsiekte sheep retrovirus and enzootic nasal tumor virus induce


