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Single residues in the surface subunits of oncogenic sheep retrovirus envelopes distinguish receptor-mediated triggering for fusion at low pH and infection

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ABSTRACT

Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus (ENTV) are two closely related oncogenic retroviruses that share the same cellular receptor yet exhibit distinct fusogenicity and infectivity. Here, we find that the low fusogenicity of ENTV envelope protein (Env) is not because of receptor binding, but lies in its intrinsic insensitivity to receptor-mediated triggering for fusion at low pH. Distinct from JSRV, shedding of ENTV surface (SU) subunit into culture medium was not enhanced by a soluble form of receptor, Hyal2 (sHyal2), and sHyal2 was unable to effectively inactivate the ENTV pseudovirions. Remarkably, replacing either of the two amino acid residues, N191 or S195, located in the ENTV SU with the corresponding JSRV residues, H191 or G195, markedly increased the Env-mediated membrane fusion activity and infection. Reciprocal amino acid substitutions also partly switched the sensitivities of ENTV and JSRV pseudovirions to sHyal2-mediated SU shedding and inactivation. While N191 is responsible for an extra N-linked glycosylation of ENTV SU relative to that of JSRV, S195 possibly forms a hydrogen bond with a surrounding amino acid residue. Molecular modeling of the pre-fusion structure of JSRV Env predicts that the segment of SU that contains H191 to G195 contacts the fusion peptide and suggests that the H191N and G195S changes seen in ENTV may stabilize its pre-fusion structure against receptor priming and therefore modulate fusion activation by Hyal2. In summary, our study reveals critical determinants in the SU subunits of JSRV and ENTV Env proteins that likely regulate their local structures and thereby differential receptor-mediated fusion activation at low pH, and these findings explain, at least in part, their distinct viral infectivity.

Introduction

Enveloped viruses encode surface glycoproteins that recognize cellular receptors and mediate viral fusion and entry into the host cells (Cosset and Lavillette, 2011). For many class I fusion proteins such as retrovirus envelope (Env), receptor binding and membrane fusion are executed by two associated subunits that are generated from proteolytic cleavage of a precursor by host proteases (White et al., 2008). The retrovirus Env glycoproteins consist of a surface (SU) and a transmembrane (TM) subunit; SU is responsible for receptor binding, and TM mediates virus-cell membrane fusion (Coffin et al., 1997). At the prefusion state, the SU subunit acts as a clamp to restrict refolding of TM and thereby prevents premature fusion activation (Colman and Lawrence, 2003; Eckert and Kim, 2001). Upon receptor binding, a conformational change occurs in the SU subunit that results in the disruption of the disulfide bond or non-covalent interactions between SU and TM; this causes the constraints of SU on TM to be released, leading to the formation of a six-helix bundle (6HB) in TM and therefore membrane fusion (Eckert and Kim, 2001; Li et al., 2008; Melikyan, 2008; Pinter et al., 1997; Wallin et al., 2004, 2006). There is also strong evidence that the retroviral SU not only prevents the refolding of TM but can also send an indispensible signal to the TM subunit, since deletion of the receptor-binding domain (RBD) of SU results in fusion-deficient Env proteins, and soluble SU proteins containing RBD are sufficient to rescue their fusogenicities *in trans* (Barnett and Cunningham, 2001; Lavillette et al., 2001).

While retroviruses are traditionally believed to fuse with host cells at the plasma membrane (McClure et al., 1990), several retroviruses have been found to require a low pH for fusion and cell entry. These include ecotropic murine leukemia virus (E-MLV) (McClure et al., 1990; Nussbaum et al., 1993), avian sarcoma and leukosis virus (ASLV) subgroups A and B (Diaz-Griffero et al., 2002; Mothes et al., 2000), mouse mammary tumor virus (MMTV) (Redmond et al., 1984; Ross et al., 2002), equine infectious anemia virus (EIAV) (Brindley and Maury, 2005; Jin et al., 2005), foamy virus (Picard-Maureau et al., 2003), as well as the oncogenic sheep retroviruses, jaagsiekte sheep retrovirus

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(JSRV) and enzootic nasal tumor virus (ENTV) that we recently reported (Bertrand et al., 2008; Côté et al., 2008a, 2008b). Among these, the ecotropic Moloney MLV (MoMLV) appears to require low pH-dependent cellular proteases for fusion activation (Kumar et al., 2007), a novel mechanism that has been recently shown to be used by several other viruses for entry (Brindley et al., 2007; Chandran et al., 2005; Huang et al., 2006; Pager and Dutch, 2005; Pager et al., 2006; Schornberg et al., 2006; Simmons et al., 2005). Notably, ASLV (Mothes et al., 2000), JSRV (Côté et al., 2009), and likely MMTV as well (Wang et al., 2008), utilize an unusual two-step mechanism, where both receptor binding and low pH are required for fusion and cell entry.

JSRV and ENTV are simple betaretroviruses that induce contagious pulmonary and nasal adenocarcinoma in sheep and goats (Fan, 2003). The Env proteins of JSRV and ENTV are active oncogenes that elicit cell transformation *in vitro* and induce tumor formation in animals (Allen et al., 2002; Caporale et al., 2006; Liu and Miller, 2007; Maeda et al., 2001; Rai et al., 2001; Wootton et al., 2005, 2006). While the mechanism underlying the tissue-specific oncogenesis by JSRV and ENTV is still currently unknown, it is likely associated with their preferential LTR promoter activities in the lung and nasal tissues (Dakessian and Fan, 2008; McGee-Estrada and Fan, 2006, 2007; Palmarini et al., 2000a). Interestingly, despite their high sequence identities at the amino acid level in the SU subunit and that both viruses use the same cellular receptor, hyaluronidase2 (Hyal2), for entry, the infectivity and host ranges of JSRV and ENTV are remarkably different (Cousens et al., 1999; Dirks et al., 2002; Rai et al., 2000, 2001). JSRV Env-pseudotyped MLV vectors transduce a wide range of cell lines, including most human, monkey, dog, cow and rabbit cells (Rai et al., 2000), whereas ENTV Env-based vectors only transduce certain sheep and human cell lines with a very low efficiency (Dirks et al., 2002). Additionally, we recently reported that ENTV Env requires an unusually low pH (<pH 4.5) for fusion as compared to that of JSRV (<pH 6.0) (Côté et al., 2008a, 2008b), and this likely contributes to the low infection rate of ENTV because of potential degradation of viral particles in the lysosome (Côté et al., 2008a).

To understand the mechanism of fusion activation and cell entry by ENTV and JSRV, we previously generated several chimeras between these two Envs, and showed that the ENTV SU subunit is primarily responsible for its low fusion activity whereas the TM subunit dictates its unusual low pH threshold (Côté et al., 2008a). Here, we aimed to further define the underlying mechanisms of the distinct fusogenicities between ENTV and JSRV Envs, and found that the relatively low fusion activity of ENTV SU is not because of poor receptor binding, but lies in its intrinsic insensitivity to receptor-mediated triggering. Our data support the notion that, while ENTV likely utilizes a similar two-step mechanism as that of JSRV for fusion and entry, there are differences in the SU subunits of these two Envs that critically regulate their distinct fusion activation.

Results

Identification of residues in the SU subunits of ENTV and JSRV Envs that determine their distinct entry efficiencies

JSRV and ENTV share the same cellular receptor, Hyal2, for entry, yet the titer of ENTV Env pseudovirions in most mammalian cells, such as HTX that express an endogenous level of Hyal2, is extremely low as compared to that of JSRV (Table 1) (Dirks et al., 2002). Remarkably, overexpression of Hyal2 in the target cells or the replacement of ENTV SU with that of JSRV dramatically rescues the ENTV titer (Table 1) (Côté et al., 2008a; Dirks et al., 2002; Van Hoeven and Miller, 2005), suggesting that ENTV SU and its interaction with Hyal2 are responsible for its low entry efficiency. Sequence comparison reveals that ENTV SU is ~94% identical to JSRV SU at the amino acid level (Cousens et al., 2004), and the region between the signal peptide and residue 204 (Fig. 1, termed putative receptor binding domain (RBD)) has been previously identified to be the major determinant for differential infectivity of ENTV and JSRV Env pseudotypes (Dirks et al., 2002; Van Hoeven and Miller, 2005). The putative RBD contains four most distinguishable changes between JSRV and ENTV, i.e., R/G-177 (R for ENTV and G for JSRV at position 177, same nomenclatures for the other mutants), T/S-180, N/H-191, and S/G-195 (Fig. 1). Among these, T/S-180 is a putative N-linked glycosylation site (N-X-S/T) common to both ENTV and JSRV, while N191 constitutes an extra N-linked glycosylation site unique to ENTV.

Table 1
Titers of MoMLV pseudovirions bearing JSRV Env, ENTV Env or their SU mutants (AP^+ foci per ml).

	Env	HTX	HTX/LH2SN
ENTV SU	WT	14 ± 8	$(2.8 \pm 1.1) \times 10^5$
	R177G	31 ± 4	$(7.7 \pm 0.1) \times 10^4$
	T180A	37 ± 3.4	$(1.8 \pm 0.2) \times 10^4$
	T180S	31 ± 3.0	$(1.9 \pm 0.8) \times 10^5$
	N191H	$\mathbf{(1.0 \pm 0.2) \times 10^3}$	$\mathbf{(1.0 \pm 0.2) \times 10^5}$
	S195G	$\mathbf{(2.0 \pm 0.3) \times 10^3}$	$\mathbf{(2.4 \pm 1.7) \times 10^6}$
	N191H/S195G	$\mathbf{(2.3 \pm 0.4) \times 10^3}$	$\mathbf{(2.7 \pm 0.6) \times 10^6}$
JSRV SU	WT	$(3.9 \pm 0.1) \times 10^4$	$(3.3 \pm 1.0) \times 10^6$
	G177R	$(7.1 \pm 0.7) \times 10^4$	$(6.6 \pm 2.1) \times 10^6$
	S180A	$(2.2 \pm 0.5) \times 10^4$	$(2.4 \pm 0.2) \times 10^6$
	S180T	$(2.3 \pm 0.2) \times 10^4$	$(1.4 \pm 1.8) \times 10^6$
	H191N	$\mathbf{(1.7 \pm 0.5) \times 10^4}$	$\mathbf{(5.3 \pm 2.2) \times 10^6}$
	G195S	$\mathbf{(2.4 \pm 1.3) \times 10^3}$	$\mathbf{(5.6 \pm 0.5) \times 10^4}$
	H191N/G195S	$\mathbf{(1.5 \pm 0.5) \times 10^2}$	$\mathbf{(7.2 \pm 1.1) \times 10^4}$

293/GP-LAPSN cells expressing the MLV Gag-Pol proteins and AP were transfected with plasmid DNA encoding individual Envs. Virions were harvested 48–72 h post-transfection, and used to infect HTX and HTX cells overexpressing Hyal2 (HTX/LH2SN). Titers were determined by counting AP^+ foci 72 h post-infection. Results are averages ± standard deviations (SD) of three independent experiments. WT: wildtype. Mutants at positions 191 and 195 and their titers are in bold.

We generated reciprocal Env mutants between ENTV and JSRV at these four positions in the RBD, and examined their effects on viral infection. The expression of these Envs and their incorporations into MLV vectors were comparable, except that ENTV Env and its mutants exhibited relatively higher levels of expression than those of JSRV as determined by flow cytometry and Western blot (data not shown; also see ref. (Côté et al., 2008a)). MLV pseudotypes bearing individual Envs were used to transduce human HTX or HTX/LH2SN cells (the latter overexpress human Hyal2), and their titers were summarized in Table 1. While reciprocal mutations between ENTV and JSRV Envs at position 177 and 180 did not significantly change the viral titers in either cell lines, substitution of ENTV S195 or N191 with the corresponding JSRV G195 or H191 increased the ENTV titer by ~10 to 100-fold in HTX and HTX/LH2SN cells (Table 1). A double mutant harboring both S195G and N191H did not appear to further increase the ENTV titer (Table 1). Interestingly, the reciprocal JSRV G195S mutant, but not that of H191N, exhibited a ~10- to100-fold decreased titer in both cell lines, and the double mutant, JSRV H191N/G195S, showed a further drop in the infection titer (Table 1).

Taken together, these results indicated that residues at position 191 and 195 are critical for ENTV and JSRV entry, with those at position 195 likely playing a more important role. We cannot rule out the possibility that other residues in the SU subunit, particularly the two additional residues in the RBD, i.e., 174 and 196, may also contribute to the differential entry efficiency of JSRV and ENTV.

The SU subunits of ENTV and JSRV Envs are differentially glycosylated

We next performed metabolic labeling assays and examined the expression, processing, and possible glycosylations of these JSRV and ENTV Env proteins and their SU mutants. As shown in Fig. 2, all the JSRV and ENTV Env constructs were expressed and processed with similar efficiencies, as evidenced by comparable band intensities for the precursors (labeled as "FL") and processed TMs (Fig. 2A).