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Short communication

Signal peptide cleavage from GP3 enabled by removal of adjacent glycosylation sites does not impair replication of equine arteritis virus in cell culture, but the hydrophobic C-terminus is essential

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ABSTRACT

The disulphide-linked GP2/3/4 spike of equine arteritis virus (EAV) is essential for virus entry. We showed recently that in transfected cells carbohydrates attached adjacent to the signal peptide of GP3 inhibit cleavage. Here we confirm this unique phenomenon in recombinant viruses with disabled glycosylation sites. Surprisingly, the infectivity of EAV containing GP3 with cleaved signal peptide was not impaired and GP3 with cleaved signal peptide associates with GP2/4 in virus particles. In contrast, viruses containing GP3 with deleted hydrophobic C-terminus rapidly reverted back to wild type. The data support our model that the signal peptide is exposed to the lumen of the ER and the C-terminus peripherally attaches GP3 to membranes.

Equine arteritis virus (EAV) is an enveloped, single-stranded, positive-sense RNA virus from the family *Arteriviridae*. EAV and another Arterivirus, porcine reproductive and respiratory syndrome virus (PRRSV) are important pathogens of domestic animals, with high economic impact. EAV can cause persistent infection in stallions that shed the virus exclusively in their semen (Balasuriya et al., 2013; Snijder et al., 2013).

Arteriviruses contain eight structural proteins: the nucleocapsid protein N and seven membrane proteins, the glycoproteins GP2, GP3, GP4, that form a disulphide-linked heterotrimeric complex in virus particles (Wieringa et al., 2003b), GP5, which is disulphide-linked to M (de Vries et al., 1995a), the small E protein (Snijder et al., 1999), a myristoylated hydrophobic protein (Thaa et al., 2009) that might function as an ion channel (Lee and Yoo, 2006) and the recently discovered membrane protein encoded by ORF5a (Firth et al., 2011; Johnson et al., 2011, see Fig. 1A). N, M and GP5 are major virion components, whereas E, GP2, GP3 and GP4 are referred to as minor structural proteins. Both in transfected and virus-infected cells, the glycoproteins are retained in the endoplasmic reticulum (ER) and/or pre-Golgi region, the viral budding site, by means of unidentified retention signals (Balasuriya et al., 2013; Snijder et al., 2013).

From reverse genetics experiments, it is known that all structural proteins are essential for virus replication. GP5 and M are needed for virus budding (de Vries et al., 1995a; Wieringa et al., 2004), whereas the GP2/3/4 spike is required for cell entry (Lu et al., 2012; Tian et al., 2012; Wieringa et al., 2004) *via* clathrin-mediated endocytosis (Nitschke et al., 2008). Interestingly, the GP2/3/4 complex interacts either physically or functionally with E. Removal of E from the viral genome completely prevents incorporation of the GP2/3/4 complex into virus particles, whereas in the absence of GP2, GP3 or GP4, the amount of E in virions is greatly reduced (Wieringa et al., 2004).

GP2 and GP4 are typical type I transmembrane proteins containing an N-terminal cleaved signal peptide and a C-terminal transmembrane region and form an intermolecular disulphide linkage in the ER (de Vries et al., 1995b; Wieringa et al., 2002, 2003a,b). GP3, the focus of this study, consists of an N-terminal uncleaved signal peptide, an ectodomain with six N-glycosylation sites, two of which are overlapping and located adjacent to the signal peptide, and a C-terminal hydrophobic region (Hedges et al., 1999a; Wieringa et al., 2002). Uniquely, disulphide linkages between GP3 and GP2/4 are formed only after release of virus particles from

Abbreviations: EAV, equine arteritis virus; ER, endoplasmic reticulum; PNGase, peptide:N-glycosidase; PRRSV, porcine reproductive and respiratory syndrome virus.

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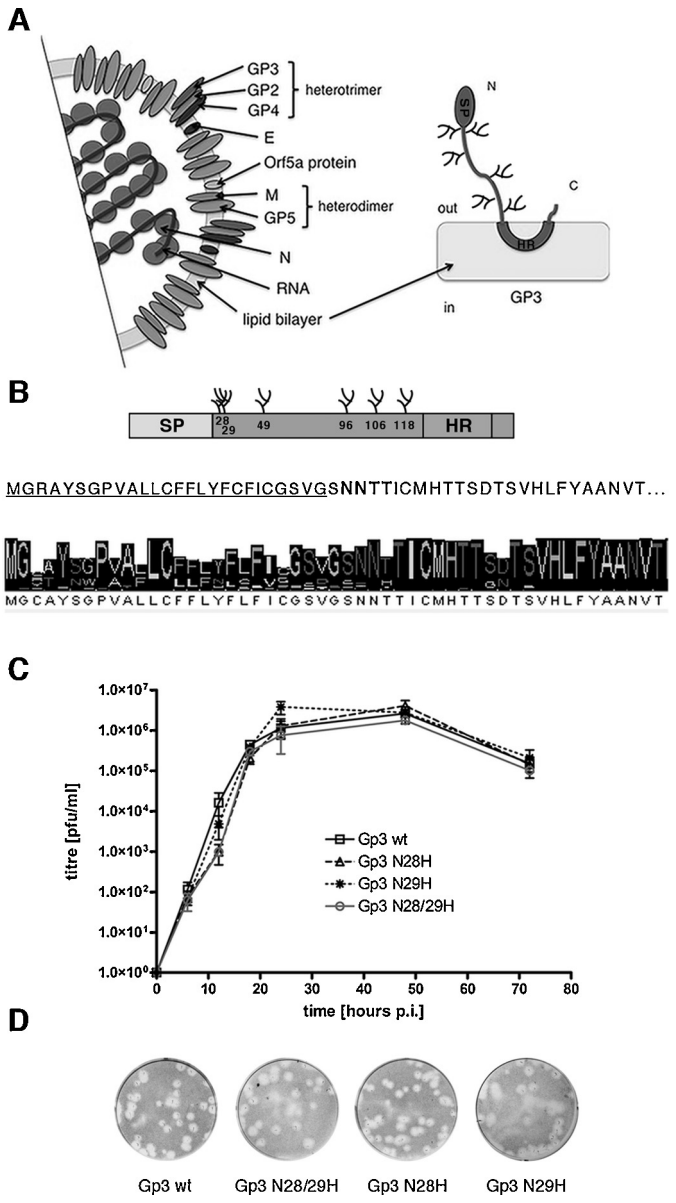

Fig. 1. Exchange of glycosylation sites adjacent to the signal peptide of GP3 does not impair growth of recombinant EAV. (A) Left part: scheme of an Arterivirus particle showing the location of the viral proteins. Right part: proposed membrane topology of GP3. The signal peptide (SP) is exposed to the lumen of the ER and the C-terminal hydrophobic region (HR) peripherally attaches GP3 to the membrane. (B) Primary structure of GP3 containing an N-terminal signal peptide and a C-terminal hydrophobic region. The location of the six glycosylation sites is depicted. The N-terminal amino acid sequence of GP3 from the Bucyrus reference strain used in this study and the relative abundance of amino acids in all EAV strains are shown below. The alignment was performed with Jalview Version 2 software (Waterhouse et al., 2009). From 249 EAV GP3 sequences extracted from NCBI, 153 (62%) contain the overlapping sequon NNTT, 93 (37%) one glycosylation site and only three no glycosylation site adjacent to the predicted signal peptide cleavage site. (C) Growth curve of wild type EAV (wt) and mutants with one (GP3-N28H, GP3-N29H) or two glycosylation sites (GP3-N28/29H) exchanged at the overlapping sequon of GP3. BHK cells were infected with an m.o.i. of 0.1, aliquots of the supernatant were collected at the indicated time points post infection (p.i.) and virus particles were titrated by plaque-assay (Thaa et al., 2009). Experiments were carried out in triplicate and titres are displayed as means ± SD. (D) Plaque assays of wild type EAV and EAV carrying the indicated mutations. Plaque assays (with agarose overlay) were stained with neutral red three days after infection.

infected cells (Wieringa et al., 2003b), but a non-covalently associated GP2/3/4 complex might form already in cells since folding of individual proteins *in vitro* is interdependent (Kabatek and Veit, 2012).

Our recent work revealed several peculiar features for processing of GP3: We demonstrated that the overlapping sequon NNTT located just downstream of the signal peptide (Fig. 1B) is efficiently (>50%) N-glycosylated at both asparagines (Matczuk et al., 2013). This is a unique observation in glycobiology, since only one asparagine per molecule is usually used in overlapping sequons (Nilsson and von Heijne, 1993). Furthermore, deletion of both (but not one) glycosylation sites of the overlapping sequon allows signal peptide cleavage, indicating that co-translational attachment of carbohydrates inhibits access of the signal peptidase to the cleavage site (Matczuk et al., 2013). This is consistent with predictions of the reliable bioinformatics tool SignalP (Petersen et al., 2011), which proposes signal peptide cleavage from GP3 with high probability, but does not take into account protein modifications like glycosylation.

Our studies also led to a new model for the membrane topology of GP3 (Fig. 1A): GP3 with uncleaved as well as with cleaved signal peptide are both membrane-bound. In contrast, deletion of the hydrophobic C-terminus of GP3 allowed secretion of the protein from transfected cells. The C-terminus of the GP3 is not a transmembrane region since it is exposed to the lumen of the ER. Thus, the hydrophobic C-terminus peripherally anchors GP3 to membranes (Matczuk et al., 2013). These results were obtained with GP3 expressed in transfected cells. Here, we analyzed whether glycosylation inhibits signal peptide cleavage also in the context of a virus infection and determined the impact of this on virus infectivity.

We first generated EAV mutants lacking either one or both glycosylation sites in the overlapping sequon NNTT of GP3 by site-directed substitution of adenine 10387 and/or 10390 by cytosine in the EAV full-length cDNA clone pEAV211 (van Dinten et al., 1997). These mutations replaced asparagines at position 28 and/or 29 by histidines without affecting the amino acid sequence of the C-terminus of GP2, which overlaps with the N-terminus of GP3 in the viral genome. BHK-21 cells were transfected with *in vitro*-transcribed full-length RNA, and virus was rescued for both wild type and mutant EAV (Thaa et al., 2009). In order to analyze the impact of the mutations on infectivity of EAV, cells were infected with recombinant virus at an m.o.i. of 0.1, cell culture medium was harvested at different time points after infection and the production of infectious progeny was analyzed by plaque assay. Surprisingly, except for a small reduction in the titre at 12 h after infection the mutant viruses reached (almost) identical titres as wild type at later time points (Fig. 1C). Likewise, the plaque size and morphology did not differ between wild type and mutants (Fig. 1D), indicating that removal of the glycosylation sites adjacent to the signal peptide in GP3 does not cause a significant growth defect in EAV.

These findings are consistent with two studies with PRRSV describing the effect of deletion of individual glycosylation sites on virus replication (Das et al., 2011; Wei et al., 2012): Recombinant viruses where asparagine 29 in GP3 (the conserved glycosylation site adjacent to the signal peptide) was exchanged grew to identical titres as wild type virus, but exhibited a slight titre reduction at early time points after infection. However, it has neither been analyzed experimentally whether GP3 of PRRSV contains an uncleaved signal peptide nor whether it is cleaved upon removal of the adjacent glycosylation site.

RNA viruses possess high mutation rates, the N-terminal region of GP3 varies between individual EAV strains (Fig. 1B) and in persistently infected carrier stallions strong selective pressure on amino acid exchange is exerted on the region encompassing the signal peptide cleavage site (Hedges et al., 1999b). Thus, the nucleotide