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Membrane Topology and Processing of the Minor Glycoproteins of Equine Arteritis Virus

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Matczuk AK, Veit M (2013) Signal peptide cleavage from Gp3 enabled by removal of adjacent glycosylation sites does not impair replication of equine arteritis virus in cell culture. *Virus Research* (submitted) – Chapter 3

Matczuk AK, Kabatek A, Veit M. Localisation of the Gp2/4 complex of EAV with exchanged potential retention signals and the effect of single cysteine mutations on the complex formation. (manuscript). – Chapter 4

I performed all experiments myself, except bioinformatic analyses on human and mouse protein databases, which was performed by Dušan Kunec (FU Berlin), and glycosidase treatment of Gp2 and Gp4 (Fig. 4.2A), which was done by Alexander Kabatek.

Table of Contents

Table of Contents 1

List of Figures... 4

List of Tables 6

Abbreviations 7

Chapter 1 10

Introduction 10

1.1 Taxonomy 10
1.2 Structure of the virion 10
1.3 Tropism and replication cycle... 11
1.4 Envelope proteins of EAV 13
1.5 Targeting membrane proteins to ER 16
1.6 Protein modification in ER: N-linked glycosylation and disulphide bond formation . 19
1.6 Trafficking and retention signals in ER 20
1.7 Aims of the study 21
1.8 References 22

Chapter 2 26

Co-translational processing of glycoprotein 3 from equine arteritis virus: 26

N-glycosylation adjacent to the signal peptide prevents cleavage 26

2.1 Abstract 26
2.2 Introduction 26
2.3 Experimental procedures 28
2.3.1 Cloning and transfection 28
2.3.2 Metabolic labeling, tunicamycin treatment, immunoprecipitation and fluorography 29
2.3.3 Glycosidase treatment 29
2.3.4 In vitro transcription/translation 30
2.3.5 Preparation of membranes and soluble fractions from microsomes 30
2.3.6 Experiments to analyze secretion of Gp3 from cells 30
2.3.7 SDS-PAGE, Western blotting, fluorography 31
2.3.8 Fluorescence protease protection assay 31
2.3.9 Bioinformatic identification of proteins that contain a glycosylation site adjacent to signal peptide cleavage site 31
2.4 Results 32
2.4.1 The overlapping sequon near the signal peptide is glycosylated at both asparagines 32
2.4.2 Removal of two glycosylation sites allows cleavage of the signal peptide 36
2.4.4 The signal peptide of Gp3 does not function as a membrane anchor 39
2.4.5 Bioinformatic analyses on the occurrence of glycosylation sites adjacent to signal peptides 45

2.5 Discussion ... 47
2.6 Acknowledgements ... 51
2.7 Footnotes ... 51
2.8 References ... 52

Chapter 3 ... 55

Signal peptide cleavage from Gp3 enabled by removal of adjacent glycosylation sites does not impair replication of equine arteritis virus in cell culture ... 55
3.1 Summary ... 55
3.2 Manuscript ... 55
3.3 Acknowledgements ... 65
3.4 Referenes ... 66

Chapter 4 ... 69

Localisation of the Gp2/4 complex of EAV with exchanged potential retention signals and the effect of single cysteine mutations on the complex formation ... 69
4.1 Abstract ... 69
4.2 Introduction ... 69
4.3 Materials and methods ... 72
4.3.1 Cells ... 72
4.3.2 Plasmids ... 72
4.3.3 Fluorescence protease protection assay ... 73
4.3.4 Metabolic labelling of proteins ... 73
4.3.5 Immunoprecipitation and glycosidase treatment ... 73
4.3.6 SDS-PAGE, Western blotting, fluorography ... 74
4.3.7 Immunofluorescence and confocal microscopy ... 74
4.3.8 Reverse genetics ... 74
4.4 Results ... 75
4.4.1 Gp2-YFP and Gp4-YFP show type I membrane topology and localise to the ER75
4.4.2 Gp2-CFP and Gp4-YFP do not pass the medial Golgi compartment ... 76
4.4.5 The cysteine residues in the ectodomain of EAV-Gp4 are essential for virus infectivity ... 82
4.5 Discussion ... 83
4.6 References ... 85

Chapter 5 ... 88

General discussion ... 88
5.1 Membrane topology of Gp3 ... 88
5.2 The hydrophobic C-terminus is responsible for membrane association of Gp3 ... 90
5.3 N-linked glycans adjacent to the signal peptide cleavage site in Gp3 interfere with cleavage ... 94
5.4 Overlapping NNTT sequon of Gp3 is glycosylated at both asparagines ... 96
5.5 The effect of N-linked glycans adjacent the SP cleavage site of Gp3 on EAV infectivity ... 97

5.6 Gp2 and Gp4 membrane topology, ER retention and dimerization 98
5.7 Final remarks and outlook 99
5.8 References.. 100
Zusammenfassung 103
Summary.. 105
Publications 107
Acknowledgements 108

List of Figures

Figure 1.1 Structure of the EAV virion 11

Figure 1.2 Genome organisation of EAV 12

Figure 1.3 Replication cycle of EAV.. 13

Figure 1.4 Kyte-Doolittle hydropathy plot of EAV Gp3. 15

Figure 1.5 The structure of a signal peptide 16

Figure 1.6 SignalP 4.0 software predictions 18

Figure 2.1 The overlapping sequon NNTT is efficiently glycosylated at both asparagines . 34

Figure 2.2 The signal peptide is cleaved from Gp3 after removal of two, but not one glycosylation site of the overlapping sequon. 37

Figure 2.3 Glycosylation of the overlapping sequon occurs rapidly and its inhibition by tunicamycin allows signal peptide cleavage 38

Figure 2.4 The C-terminus of Gp3 is translocated into the lumen of the ER 40

Figure 2.5 Signal peptide cleavage does not convert Gp3 into a soluble protein. 41

Figure 2.6 Deletion of the C terminus allows secretion of Gp3 from transfected cell 43

Figure 2.7 Model for the membrane topology of Gp3. 44

Figure 3.1 Exchange of glycosylation sites adjacent to the signal peptide of Gp3 does not impair growth of recombinant EAV. 58

Figure 3.2 EAV containing Gp3 with cleaved signal peptide has no fitness defect, but recombinant virus containing Gp3 with deleted C-terminus could not be generated.. 60

Figure 3.3 Removal of glycosylation sites does not affect the protein composition of EAV particles.. 63

Figure 4.1 The C-terminus of Gp2 and Gp4 is not translocated into the lumen of the ER... 75

Figure 4.2 Gp2-CFP and Gp4-YFP do not pass medial Golgi compartment. Gp4-YFP contains three N-linked glycans 76

Figure 4.3 Schematic picture of the Gp2 and Gp4 constructs used in the study 77

Figure 4.4 Dimerisation and subcellular localisation of Gp2 and Gp4 dimers with exchanged TMD and cytoplasmic tails. 79

Figure 4.5 The single cysteine mutations in Gp4 do not influence Gp2-HA/Gp4-Flag dimerization.. 81

Figure 4.6 Gp4 cysteine mutants virus rescue and plaque morphology. 82

Figure 5.1 Helical wheel plot analysis of the C-terminal hydrophobic part of EAV Gp3 90

Figure 5.2. Kyte-Doolittle hydrophaty plot of Gp3 from all *Arteriviridae* family members.. 92

Figure 5.3 Model of interaction of Gp3 with the translocon and associated complexes....... 95

List of Tables

Table 2.1 Amino acid sequences and signal peptide cleavage site prediction for Gp3 sequences. 35

Table 2.2 N-terminal sequence of Gp3 of EAV: glycosylation prevents signal peptide cleavage..... 45

Table 2.3 N-terminal sequence of proteins for which it has been experimentally shown that efficient N-glycosylation in the neighborhood of the signal peptide requires cleavage 46

Table 2.4 Human proteins containing an N-glycosylation site downstream of the predicted (but not experimentally verified) signal peptide cleavage site 46

Table 5.1 The comparison of the Gp3 in *Arteriviridae* 93

Abbreviations

^{35}S	Sulfur 35
BHK-21	Baby hamster kidney cells
C	Carboxyl terminus
cDNA	Complementary DNA
CFP	*Cyan fluorescent protein*
CHO-K1	Chinese hamster ovary cells
DMV	Double membrane vesicle
E	Envelope protein
EAV	Equine arteritis virus
Endo H	Endoglycosidase H
ER	Endoplasmatic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
ERGIC	ER-Golgi intermediate compartment
GFP	Green fluorescent protein
Gp2	Glycoprotein 2
Gp3	Glycoprotein 3
Gp4	Glycoprotein 4
Gp5	Glycoprotein 5
HA	Haemagglutinin
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HR	Hydrophobic region
IBV	Avian infectious bronchitis virus
IP	Immunoprecipitation
kDa	Kilo Dalton
LDV	Lactate dehydrogenase-elevating virus
M	Membrane protein
m.o.i.	Multiplicity of infection
MHC	Major histocompatibility complex
mRNA	Messenger RNA
N	Nucleocapsid protein

N	Amino terminus
NA	Neuraminidase
NCBI	National Center for Biotechnology Information
nsp	Non-structural protein
ORF	Open reading frame
OST	Oligosaccharyl transferase
p.i.	Post infection
PBS	Phosphate-buffered saline
PNGase F	Peptide-n-glycosidase
pp	Polyprotein
PRRSV	Porcine reproductive and respiratory syndrome virus
PVDF	Polyvinylidene difluoride
RIPA	Radioimmune precipitation assay buffer
RK-13	Rabbit kidney cells
RTC	Replication and transcription complex
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SFV	Semliki forest virus
sgRNA	Subgenomic RNA
SHFV	Simian hemorrhagic fever virus
SP	Signal peptide
SPase	Signal peptidase
SPC	Signal peptidase complex
SPP	Signal peptide peptidase
SR	Signal recognition particle receptor
SRCR	Scavenger receptor cysteine-rich
SRP	Signal recognition particle
TM	Transmembrane
TMD	Transmembrane domain
TMR	Transmembrane region
TRS	Transcription regulatory sequences
US11	Unique short 11 protein
VERO	African green monkey kidney

WB	Western blot
wt	Wild type
YFP	Yellow fluorescent protein

Chapter 1

Introduction

Equine arteritis virus (EAV) is the prototype member of family *Arteriviridae* and the causative agent of equine viral arteritis, a disease affecting horses and donkeys worldwide. EAV causes abortions in pregnant mares and respiratory illness with flu-like symptoms, which in young animals can cause death. The virus is transmitted via the respiratory route and through contaminated semen of persistently infected stallions. Despite available vaccines, EAV remains an important pathogen in the horse industry, as outbreaks continue to occur and new strains are arising (MacLachlan and Balasuriya, 2006).

1.1 Taxonomy

Arteriviridae, together with *Coronaviridae*, *Toroviridae* and *Roniviridae*, belong to order Nidovirales, which are enveloped viruses with a positive sense, single stranded RNA genome. Besides EAV, the other members of *Arteriviridae* are porcine reproductive and respiratory syndrome virus (PRRSV), murine lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV). The PRRSV has two genotypes, North American (type 2) and European (type 1) (Snijder *et al.*, 2013).

1.2 Structure of the virion

EAV particles are round, "smooth" and have a diameter of approximately 65 nm (Magnusson *et al.*, 1970). The genome consists of 12,7 kb single RNA molecule of positive polarity coupled to 14 kDa phosphorylated nucleocapsid protein N, the product of ORF7. The envelope of EAV consists of non-glycosylated proteins E and M and glycoproteins Gp2, Gp3, Gp4 and Gp5 (Balasuriya *et al.*, 2013). The newly discovered Orf5a protein is probably another structural protein of EAV virion (Firth *et al.*, 2011).

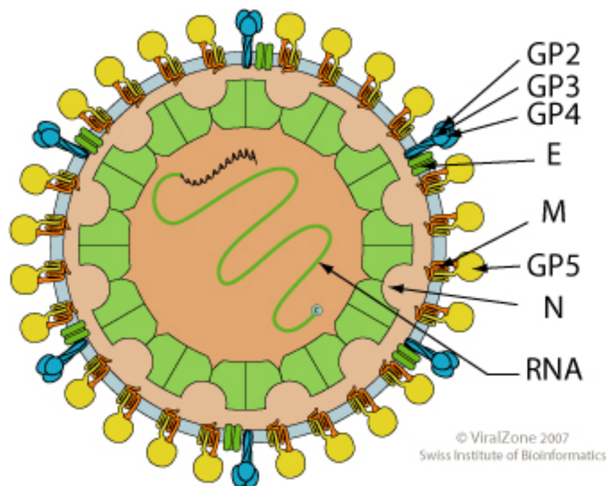

Figure 1.1 Structure of the EAV virion. Gp: glycoprotein; E: envelope protein; M: membrane protein; N: nucleocapsid protein. Used with permission from Swiss Institute of Bioinformatics. Source: ViralZone: www.expasy.org/viralzone.

1.3 Tropism and replication cycle

EAV infects primarily cells from the monocyte lineage (macrophages, monocytes), endothelial cells of small blood vessels, some epithelial cells, mesothelium and smooth muscle cells of the tunica media of small arteries. Later during viraemia, virus spreads to multiple tissues, infecting endothelial and epithelial cells of lymphoid organs, lungs, intestines, kidneys, placenta and male accessory genital glands (Balasuriya *et al.*, 2013; MacLachlan *et al.*, 1996). Unlike other Arteriviruses, EAV exhibits a wide tropism in cell culture, including primary cells and continuous cell lines.

EAV enters the cell via clathrin-mediated endocytosis (Nitschke *et al.*, 2008). Upon acidification, the viral membrane fuses with the membrane of the early endosome, but the details of this process as well as the cellular receptor are unknown. Replication takes place in perinuclear replication centers characterised by double-membrane structures, which are induced upon infection (Knoops *et al.*, 2012).

The genome of EAV is characterized by nested genome, meaning that most of the ten known open reading frames (ORFs) overlap with each other (Fig. 1.2). The major part of the genome is occupied by the 5` terminal ORFs 1a and 1b, encoding mainly replicative enzymes. The products of these ORFs are two long non-structural proteins pp1a and pp1ab respectively, which are precursors for at least 13 non-structural proteins (nsp), processed by three viral proteinase domains encoded by ORF1a (Fang and Snijder, 2010). The nsps

assemble into membrane-associated complexes, which perform and regulate replication and transcription. The replication complex is translated directly from the parental +RNA, while the new genome copies are synthesised from –RNA intermediate.

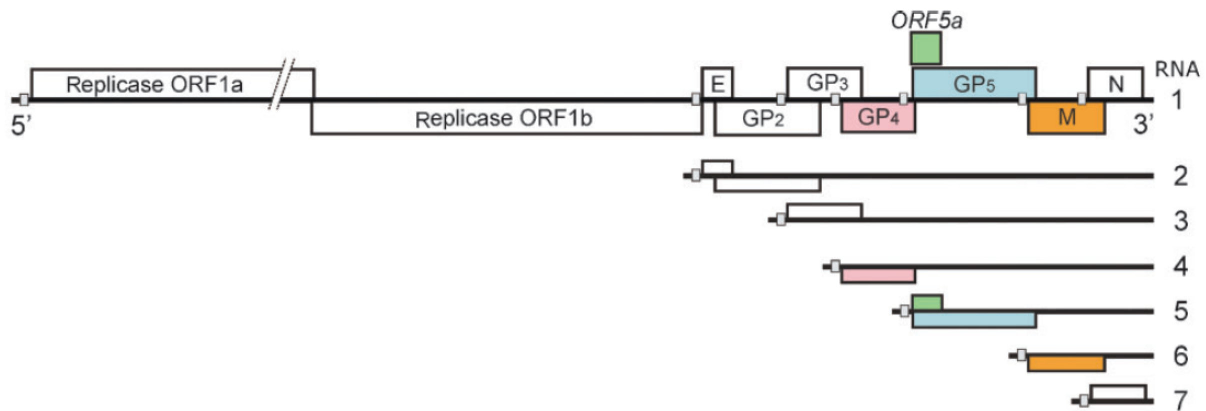

Figure 1.2 Genome organisation of EAV. The ORF1a and ORF1b encode non-structural proteins. The 3' end encodes structural genes, which are transcribed as six subgenomic RNAs. Small squares represent transcription regulatory sequences. Reproduced with permission from Firth *et al.,* J Gen Virol 92 (2011) 1097–1106.

The structural proteins are encoded by ORFs 2–7, which partially overlap with neighbouring genes. The ORFs 2–7 are first transcribed as minus-sense 3` terminal subgenomic RNAs (sgRNAs), which are regulated by transcription regulatory sequences (TRSs), leading to production of varied amounts of sgRNAs of different lenghts which are later the templates for the mRNAs encoding viral structural proteins (Pasternak *et al.*, 2006; Sawicki *et al.*, 2007). These mRNAs are monocystronic, except for mRNA2 and mRNA5, which are bicistronic, due to leaky scanning phenomenon, and encode E, Gp2, Orf5a and Gp5 (Snijder *et al.*, 2013).

Membrane proteins are targeted to the endoplasmatic reticulum (ER). Virus assembles in the internal membranes. Then virions travel through secretory pathway, and are released by exocytosis (Wada *et al.*, 1995) (Fig. 1.3).

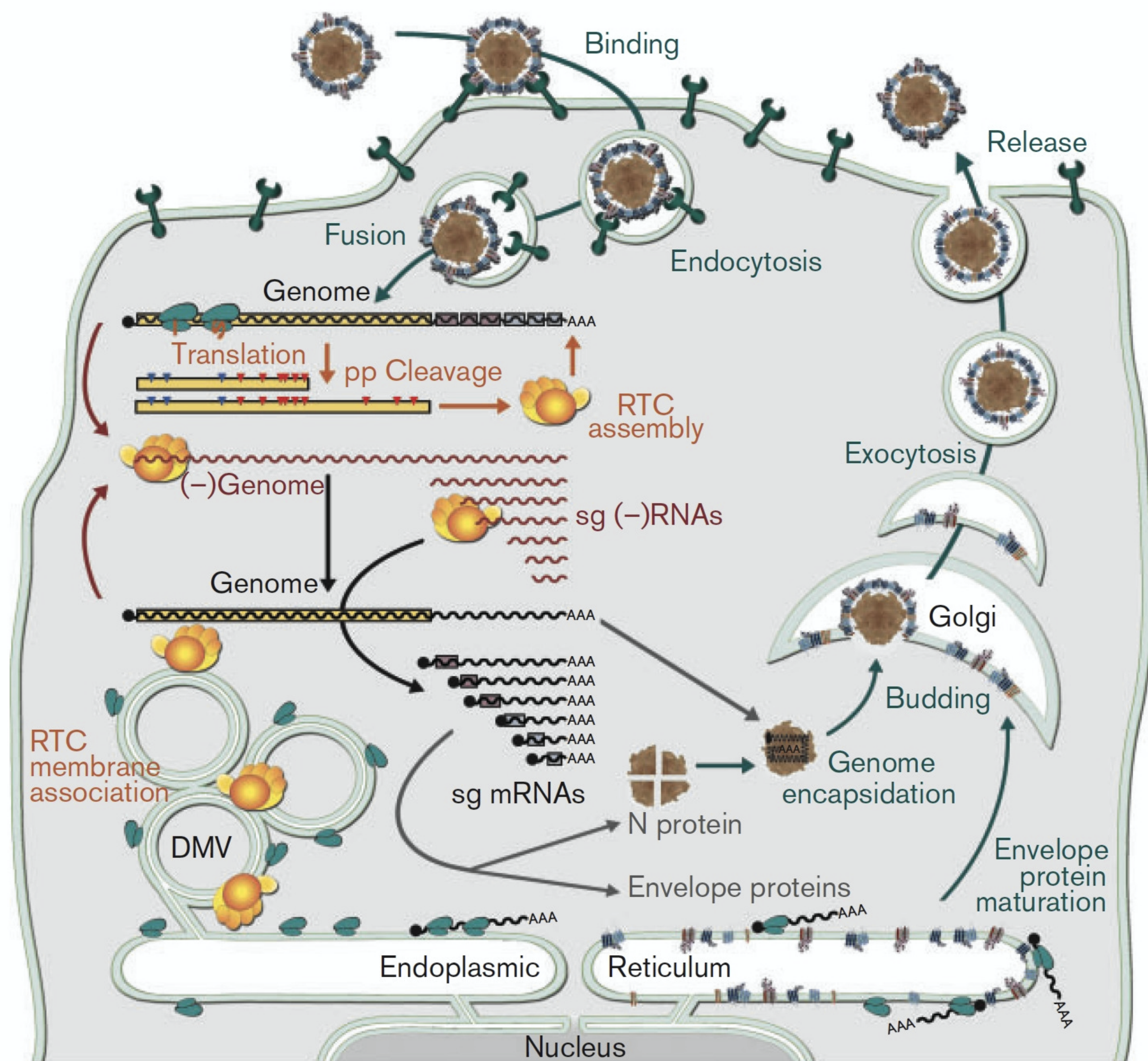

Figure 1.3 Replication cycle of EAV. pp – polyprotein; RTC – replication and transcription complex; sg – subgenomic; DMV – double membrane vesicle. Adapted with permission from Snijder *et al.*, J Gen Virol 94 (2013) 2141–2163.

1.4 Envelope proteins of EAV

The major envelope proteins consist of the glycoprotein Gp5 and the non-glycosylated protein M. These proteins are the most abundant fraction of the viral envelope. The glycoprotein Gp5, encoded by ORF5, has a size of 30 to 45 kDa, possesses cleavable signal peptide (SP) and three membrane-spanning domains. The 16 kDa membrane protein M, encoded by ORF6, has three potential membrane spanning domains. Gp5 and M form a disulphide-linked heterodimer (de Vries *et al.*, 1995a).

The N coupled to RNA, Gp5 and M are minimal requirements for formation of viral particles, therefore their main function is assembly and budding (Wieringa *et al.*, 2004). The other known function of Gp5 and M lays in cell entry. For the related Arterivirus PRRSV it was shown, that the Gp5/M dimer interacts with heparan sulphate and sialoadhesin on the cell surface (Delputte *et al.*, 2002; Vanderheijden *et al.*, 2003). The binding to sialodhesin on the surface of alveolar macrophages, a primary target for PRRSV, induce internalization of the virion (Van Breedam *et al.*, 2010).

The minor envelope proteins are the small protein fraction of the virion. Three minor membrane glycoproteins, products of ORFs 2b–4, are present in virions as disulphied-linked Gp2/Gp3/Gp4 heterotrimers, and to a lesser extent as Gp2/Gp4 heterodimers. The Gp2 and Gp4 form a covalently linked heterodimer already in the infected cell, whereas Gp3 associates with Gp2/Gp4 dimer shortly after virion release (Wieringa *et al.*, 2003a; Wieringa *et al.*, 2003b).

The Gp2 is a 227 amino acid conventional class I integral membrane protein containing an N-terminal cleavable signal peptide, an ectodomain with one potential glycosylation site, a C-terminal transmembrane domain (TMD) and a cytoplasmic tail (de Vries *et al.*, 1995b). In the Gp2 cysteine residues at amino acid positions 48 and 137 are responsible for the formation of an intramolecular covalent bond within Gp2, while the cysteine 102 forms an intermolecular bridge to Gp4 (Wieringa *et al.*, 2003a).

The Gp3 is a 163 amino acid integral membrane protein, with two main hydrophobic domains: a signal peptide (SP) and a C-terminal hydrophobic region (HR) (Fig.1.4A). The topology of Gp3 remains uncertain; it was proposed that Gp3 could be anchored to membranes via uncleaved SP or via SP and C-terminal HR (Fig. 1.4B a and b) (Wieringa *et al.*, 2002). However, bioinformatics SP cleavage prediction (see Fig. 1.6 for details) predicts a Gp3 as type I membrane protein with cleavable SP (Fig.1.4B–c). Gp3 possesses six potential N-glycosylation sites (consensus NXT/S where X is any amino acid except proline); the first two are overlapping (NNTT). The topology of the Gp3 and its processing will be the main subject of this thesis.

The Gp4 is a 152 amino acid long class I membrane protein, with cleavable SP, an ectodamain containing 4 potential N-glycosylation sites and a short cytoplasmic tail (Wieringa *et al.*, 2002). Mentioned knowledge about the topology of minor glycoproteins of EAV comes from proteins synthetised *in vitro* in presence of canine microsomal membranes as a source of ER.

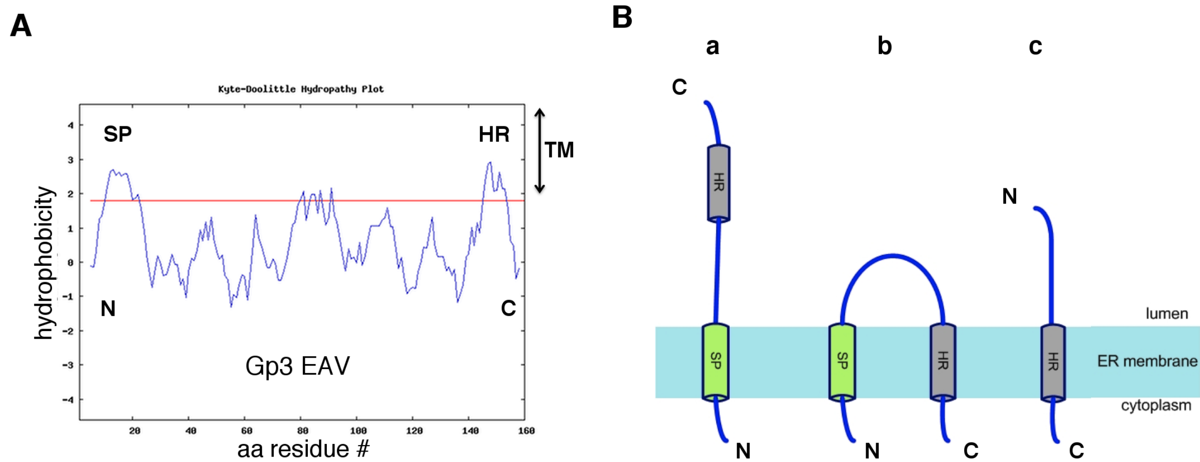

Figure 1.4 (A) Kyte-Doolittle hydropathy plot of EAV Gp3. y-axise: hydropathy score, x-axis: amino acid position. Red line indicates a threshold of transmembrane (TM) domain prediction (http://gcat.davidson.edu/DGPB/kd/kyte-doolittle.htm). **(B)** Possible membrane topologies of Gp3. Two first models adapted from Wieringa *et al.,* 2003a. SP: signal peptide; HR: hydrophobic region, N: amino terminus, C: carboxyl terminus.

The small, 8 kDa non-glycosylated E is presumed, at least for PRRSV, to act as an ion channel (Lee and Yoo, 2006). The N-terminus of E is myristoylated, and was shown to be important for virus infectivity (Thaa *et al.*, 2009). The E protein is also involved in incorporation of Gp2, Gp3 and Gp4 into virions, as deletion of E rendered the particles uninfectious and led to non-incorporation of other minor envelope proteins (Wieringa *et al.*, 2004).

All of mentioned membrane proteins of EAV (except recently discovered Orf5a) are essential for virus infectivity. The intact Gp2/Gp3/Gp4 trimer, together with E protein, is essential for virus infectivity, as knocking down each of minor glycoprotein separately, yielded non-infectious viral particles consisting of RNA, N, M and Gp5 (Wieringa *et al.*, 2003a; Wieringa *et al.*, 2004). Mutations of the cysteine residues in Gp2, which disrupted the trimer, were also lethal for the EAV (Wieringa *et al.*, 2003a).

In PRRSV Gp2 and Gp4 were shown to associate with CD163, a scavenger receptor. Expressing CD163 in non-permissive cell lines, allowed productive infection with PRRSV, suggesting that CD163 is a receptor for this virus (Calvert *et al.*, 2007). Recently, a chimeric Arterivirus composed of ORF1 and ORF5–7 from PRRSV with ORF2–4 (encoding E, Gp2,

Gp3 and Gp4) from EAV, was shown to replicate in cells that are permissive for EAV but not for PRRSV. This reveals that indeed the minor envelope proteins are responsible for cell tropism of Arteriviruses (Tian *et al.*, 2012).

1.5 Targeting membrane proteins to ER

The viral envelope proteins must enter the ER for membrane insertion, co- and post-translational modifications such as signal peptide cleavage, glycosylation, disulphide bond formation and interactions with chaperons. All these events are important for protein membrane topology, correct folding, oligomerisation and trafficking to the site of viral budding (Braakman and Bulleid, 2011). In order to gain access to a particular membrane compartment in the cell a protein has to possess a targeting sequence.

Signal peptides (SP) are N-terminal parts of proteins that target the polypeptide that is being translated from cytosol to the ER. All membrane proteins and secreted proteins contain signal peptides. There is no consensus sequence for the SP, but a consensus overall structure. The SP is approximately 20–30 amino acid long (but might be up to 80 amino acids), with a typical three domain composition: an n-region consisting of basic amino acids, a middle hydrophobic part h, and a slightly polar c-region (Fig 1.5) (Auclair *et al.*, 2012).

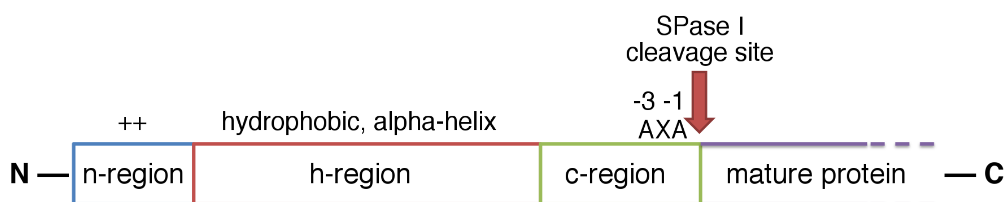

Figure 1.5 The structure of a signal peptide. The n-region possesses positively charged residues (blue), the h-region contains hydrophobic residues (red), and the c-region is typically neutral and contains the cleavage site with consensus AXA, where A is alanine or other small neutral residue and x is any amino acid. N: amino terminus; C: carboxyl terminus. Adapted with permission from Auclair *et al.*, Protein Science 21 (2012) 13–25.

In higher eukaryotes only co-translational translocation into the ER is observed. The SP emerging from the ribosome is recognised by signal recognition particle (SRP), a ribonucleoprotein that arrests translation and drives the ribosome to the ER. The SRP with bound ribosome interacts with its cognate receptor located in the ER membrane (Nyathi *et al.*, 2013). Later, the ribosome-SRP-receptor complex associates with the translocation channel. This channel, called in eukaryota the Sec61 complex, is a heterotrimer, where one subunit builds a pore closed with a plug. This plug is assumingly displaced by the binding of ribosome-nascent chain complex to the translocon (Nyathi *et al.*, 2013). Simultaneously the SP associates with the translocon and probably surrounding lipids, and the newly synthetised amino acid chain is translocated into the lumen of ER through the central aqueous pore until the downstream hydrophobic region enters the translocon. The TMDs are believed to adopt the α-helical structure before emerging from the ribosome. Such helices associate with the translocation channel in a way that opens it on one side to allow interaction of the TMD with the lipid bilayer. This mechanism known as lateral gating is still not completely investigated and understood (Auclair *et al.*, 2012; Nyathi *et al.*, 2013).

In addition, other complexes have been observed to associate with Sec61 during translocation. This includes enzymes responsible for co-translational modifications, such as oligosaccharyl transferase (OST) and signal peptidase (SPase), as well as proteins which assist the translocation of some but not all proteins, and have yet undefined functions (Nyathi *et al.*, 2013).

Most of the SPs are absent in the mature protein, because there are cleaved off by translocon-associated SPase. This enzyme is a serine protease and forms a complex called the signal peptidase complex (SPC) located on the luminal side of the ER membrane. SPC is a hetero-oligomeric membrane protein, which in mammals contains five subunits (Shelness *et al.*, 1993). The most-studied bacterial homologue SPase I recognises and cleaves signal peptides with consensus motif alanine-X-alanine, in the c-region of the SP (Fig.1.5). In eukaryotes the –1 and –3 amino acid residues upstream from cleavage site are typically small neutral residues such as alanine, glycine, cysteine and serine (Auclair *et al.*, 2012).

The timing of SP cleavage is hard to estimate, but it is assumed to occur co-translationally, meaning while the translocation of the protein chain is still in progress. However, some signal sequences seem to regulate the timing of cleavage, for instance the

exceptionally slow cleavage of the human immunodeficiency virus (HIV) gp120 SP remains uncleaved if disulphide bond formation is inhibited (Land *et al.*, 2003).

The cleaved signal peptides are processed by signal peptide peptidases (SPP). These are a group of several aspartyl proteases cleaving within the membrane of ER (Paetzel *et al.*, 2002). The cleaved SPs are translocated to the cytosol, where they are further degraded.

Some signal peptides are not cleaved, and remain as a part of the mature protein. As they are hydrophobic they usually form a TMD. Such SPs are named signal anchors, and are typical or even define the type II membrane topology (see also Fig.1.4B–a).

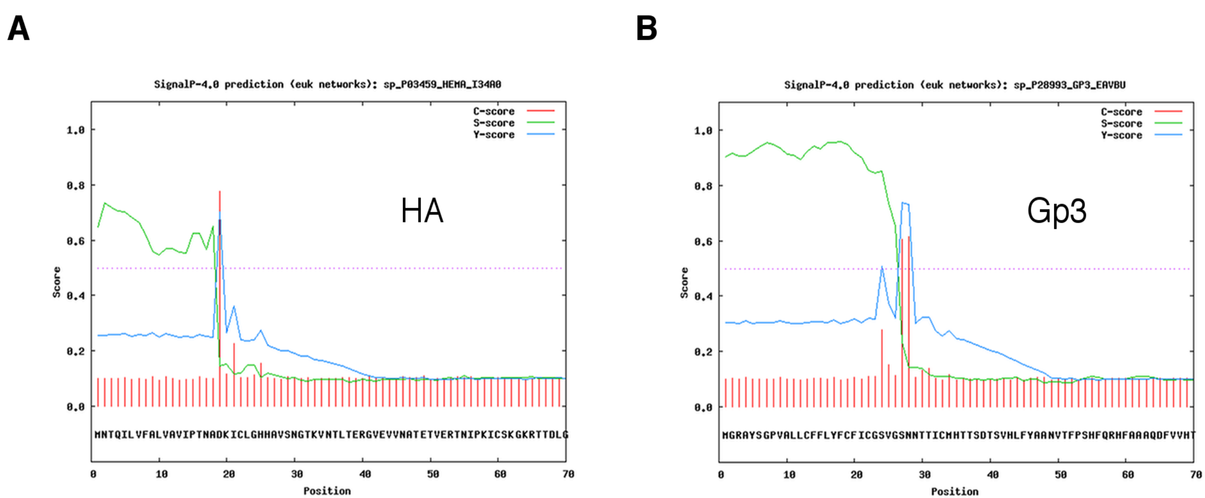

Figure 1.6 SignalP 4.0 software predictions (http://www.cbs.dtu.dk/services/SignalP-4.0/) for **(A)** haemagglutinin (HA) of the Influenza A virus (strain A/Fowl plague virus/Rostock/8/1934 H7N1) and **(B)** Gp3 of EAV. Position: amino acid position; C-score (red vertical lines): signal peptide cleavage score calculated for each amino acid; S-score (green line): signal peptide score **(**high scores indicate that the corresponding amino acid is part of a signal peptide, and low scores indicate that the amino acid is part of a mature protein); y-score (blue line): combined C and S scores, shows the most probable SP cleavage site.

On the basis of amino acid sequences of known signal peptides, several prediction software were developed, SignalP being the most reliable (Petersen *et al.*, 2011). SignalP calculates the probability of particular sequence to be a signal peptide and gives the output of the most probable SP cleavage site. In figure 1.6 the SignalP 4.0 output is presented. The

SP of haemagglutinin of Influenza A and Gp3 of EAV are predicted to be cleaved between amino acid position 18–19 and 26–27 respectively. However, as mentioned earlier, the Gp3 of EAV seems to have an uncleaved SP, therefore it is possible that different or unusually event occurs during its translation and translocation into ER (Wieringa *et al.*, 2002).

1.6 Protein modification in ER: N-linked glycosylation and disulphide bond formation

N-linked glycosylation, in which a high-mannose core is attached to the amide nitrogen of asparagine in the context of the conserved motif N–X–T/S (asparagine–any amino acid except proline– threonine or serine), is the most abundant protein modification found in eukaryotes. In case of viral glycoproteins, N-linked glycosylation is important not only for correct protein folding and subsequent intracellular trafficking, but also for a variety of functions, such as receptor binding, viral membrane fusion, virulence and immune evasion (Vigerust and Shepherd, 2007). The EAV possesses four different glycoproteins, but little is known about impact of the glycosylation on the virus infectivity. In PRRSV depending on protein and glycan localisation, some of the sites are dispensable and some crucial for virus replication in cell culture, as well as for immune evasion in the host (Ansari *et al.*, 2006; Wei *et al.*, 2012).

The glycan assembly takes place at cytosolic and later luminal part of the ER and result in the formation of a tetradecasaccharide ($Glc_3Man_9GlcNAc_2$ – Glc: glucose; Man: mannose; GlcNAc: N-acetyloglucosamine) linked to membrane-bound dolichyldiphosphate carrier. The N-glycosylation process occurs at the membrane of ER, and is performed by the multimeric oligosaccharyl transferase (OST) complex. In mammals the OST comprises at least seven membrane-bound subunits (Kelleher and Gilmore, 2006). Recent findings indicate that multiple forms of OST complex containing various combinations of subunits exist within the same cell and may modify the activity and specificity of N-linked glycan attachment (Larkin and Imperiali, 2011). The STT3 subunit contains the active site of OST (Nilsson *et al.*, 2003). Insects and vertebrates encode two isoforms, STT3A and STT3B, which have different modes of action. The OST complex with the STT3A isoform associates with the Sec61 translocon and glycosylates in a co-translational manner. STT3B, which is less efficient and probably not translocon-associated, can mediate post-translational modification of skipped glycosylation sites in unfolded proteins (Ruiz-Canada *et al.*, 2009). It was experimentally shown that the STT3A complex skips sequons located within ~70 C-terminal amino acid residues of a glycoprotein or sequons located near the SP cleavage site,

which can be modified after the SP cleavage. Such skipped sequons can be modified by STT3B (Shrimal *et al.*, 2013). In general, the NXT sequons are used more efficiently then NXS (Zielinska *et al*., 2010).

Another important protein modification occurring in ER is disulphide bond formation. Disulphide bonds are formed between the thiol groups of cysteine residues. Intrachain disulphide bonds are formed between two cysteines within the same protein chain, whereas interchain disulphide bonds link homo- and hetero-oligomers. In eukaryotes, the disulphide bond formation takes place almost exclusively in the ER by disulphide exchange with a member of the protein disulphide isomerase family of oxidoreductases. Native disulphide bond formation is a complex process, not only must disulphide bond be formed (oxidation), but incorrect bonds must be broken (reduction) or rearranged (isomerization) also (Ellgaard and Ruddock, 2005). The Gp2, Gp3 and Gp4 of EAV form covalently linked trimer, but it is unknown which cysteine in Gp4 and Gp3 participate in covalent bond formation within the complex.

1.6 Trafficking and retention signals in ER

N-glycosylation and disulphide bond formation influence proper folding and oligomerisation of proteins. If the protein is misfolded it is retained in the ER and targeted for degradation through ERAD (endoplasmic-reticulum-associated protein degradation), translocated back to the cytosol, ubiquitinated and degraded by proteasome. Only correctly folded and oligomerised proteins can leave the ER by vesicular transport through ERGIC, individual compartments of Golgi (cis, medial and trans) and later to the plasma membrane (Braakman and Bulleid, 2011).

Most of the membrane proteins have defined localization. The minor envelope proteins of EAV are never present on plasma membrane, and when expressed separately always are retained in ER, despite absence of any known retention motifs (Snijder *et al.*, 2013). The most known and studied ER retention signal of membrane proteins is the C-terminal di-lysine motif: K(X)KXX. The other known motifs of ER retention are arginine motifs. The retention of proteins with such motifs was proposed to occur via exclusion from transport vesicles or retrograde transport from the Golgi to the ER (Teasdale and Jackson, 1996). However, many ER- and Golgi-resident proteins do not possess any retention motif. Another described model of retention relies on the length of TMDs. Bioinformatics analysis

of TMDs of membrane proteins localized in different cellular membranes revealed that the length of TMDs reflects the thickness of phospholipid bilayer in particular compartment, i.e. TMDs of plasma membrane expressed proteins are longer than those of Golgi or ER (Sharpe *et al.*, 2010). As the minor enveloped proteins of EAV do not contain any known retention motif, their cellular localisation might be dependent on the length of TMD.

1.7 Aims of the study

The minor envelope proteins of Arteriviruses gained more attention in recent years due to the discovery of the PRRSV receptor CD163 and subsequent studies indicating that this receptor interacts with Gp2 and Gp4 (Calvert *et al.*, 2007; Das *et al.*, 2010). As the minor proteins are dispensable for virus assembly and budding, but essential for virus infectivity, it was first assumed and then experimentally proven that they are responsible for cellular tropism of Arteriviruses (Tian *et al.*, 2012).

In spite of their important role in virus entry, there is little data available about arteriviral minor glycoproteins. The membrane topology, for instance, was only investigated in *in vitro* systems with canine microsomal membranes, but not in transfected or infected cells.

In this thesis, I aimed to understand the processing of minor glycoproteins, with emphasis on Gp3. Previously published *in vitro* studies indicated that uncleaved SP of Gp3 may act as a membrane anchor alone, or that protein is anchored to membrane via N- and C-termini. The aim was to experimentally confirm the topology model of Gp3, and address which part of the protein is responsible for its anchoring with the membrane. The other aim was to investigate the impact of the N-linked glycosylation adjacent to predicted SP cleavage site on the retention of SP in Gp3. Does the presence of the glycans just downstream cleavage site interfere with SP cleavage in Gp3? What are possible implications for EAV infectivity?

The Gp2/Gp3/Gp4 trimeric complex is a likely candidate to perform receptor binding and fusion of Arteriviruses, but it is very difficult to study its function due to ER retention, formation of complex in ER and extracellular, small amounts of complex in the virions, lack of good antibodies, and limitations of reverse genetic system due to nested genome. Therefore, another aim of my work was to express the minor glycoprotein complex on the plasma membrane. Such expression would be a very promising tool for future functional studies and the role of the Gp2/Gp3/Gp4 trimer in Arteriviruses. Also, the

oligomerisation between Gp2 and Gp4 was found to be crucial for virus infectivity (Wieringa *et al.*, 2003a). As the cysteine responsible for the covalent bond between these two glycoproteins is only known for the Gp2, another aim was to investigate the partner cysteine in the Gp4.

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Chapter 2

Co-translational processing of glycoprotein 3 from equine arteritis virus: N-glycosylation adjacent to the signal peptide prevents cleavage

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2.1 Abstract

Signal peptide cleavage and N-glycosylation of proteins are co-translational processes, but little is known about their interplay if they compete for adjacent sites. Here we report two unique findings for processing of glycoprotein 3 of equine arteritis virus. Glycoprotein 3 (Gp3) contains an N-terminal signal peptide, which is not removed although bioinformatics predicts cleavage with high probability. There is an overlapping sequon NNTT adjacent to the signal peptide, which we show to be glycosylated at both asparagines. Exchanging the overlapping sequon and blocking glycosylation allows signal peptide cleavage, indicating that carbohydrate attachment inhibits processing of a potentially cleavable signal peptide. Bioinformatic analyses suggest that a similar processing scheme may exist for some cellular proteins. Membrane fractionation and secretion experiments revealed that the signal peptide of Gp3 does not act as a membrane anchor indicating that it is completely translocated into the lumen of the ER. Membrane attachment is caused by the hydrophobic C-terminus of Gp3, which however does not span the membrane, but rather attaches the protein peripherally to endoplasmic reticulum membranes.

Chapter 3

Signal peptide cleavage from Gp3 enabled by removal of adjacent glycosylation sites does not impair replication of equine arteritis virus in cell culture

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3.1 Summary

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 deleted 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.

Keywords: Arterivirus, equine arteritis virus, Gp3, signal peptide, glycosylation, membrane topology

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

Chapter 4

Localisation of the Gp2/4 complex of EAV with exchanged potential retention signals and the effect of single cysteine mutations on the complex formation

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4.1 Abstract

The equine arteritis virus (EAV) minor glycoproteins Gp2 and Gp4 form a disulphide-linked dimer, which in the released virion associates with Gp3. This trimer is essential for virus infectivity and cell tropism. In this paper, we confirm the type I membrane topology of Gp2 and Gp4 and the number of N-linked glycans (1 and 3, respectively). The Gp2 and Gp4 tagged with YFP were retained in ER. In order to express the Gp2/4 dimer on the plasma membrane for functional studies, the transmembrane regions and cytoplasmic tails of Gp2 and Gp4 were exchanged with corresponding regions of influenza A haemagglutinin. The proteins expressed together were able to form a Gp2/4 heterodimer, which partially co-localised with cis-Golgi marker, but was not targeted to plasma membrane. When individual cysteines in the ectodomain of Gp4 were mutated, the Gp4 still formed a disulphide-linked dimer. More experiments are needed to reveal the nature of the covalent linkages in the Gp2/4 dimer, and why the complex is retained in the internal membranes.

4.2 Introduction

Equine arteritis virus (EAV) is an enveloped, single strand, positive sense RNA virus from the family *Arteriviridae*, order Nidovirales, which is responsible for abortion and respiratory disease in horses worldwide. EAV is the prototype Arterivirus, the other members of this family include porcine reproductive and respiratory syndrome virus (PRRSV), lactate dehydrogenase-elevating virus (LDV) and simian haemorrhagic fever virus (SHFV).

The EAV virion consists of nucleocapsid protein N coupled to RNA, and at least six different membrane proteins. The glycoprotein Gp5 and the membrane protein M form a disulphide-linked heterodimer, these complexes are the major protein fraction of the mature virion (Balasuriya *et al.*, 2013). The remaining membrane proteins are present in small amounts in the virion and are named "minor envelope proteins": the small envelope protein E, and the glycoproteins Gp2, Gp3 and Gp4. All the mentioned structural proteins are essential for virus infectivity (Wieringa *et al.*, 2004). It was previously shown that N, Gp5 and M are responsible for budding, whereas E, Gp2, Gp3 and Gp4 are responsible for cell tropism (Wieringa *et al.*, 2004; Tian *et al.*, 2012).

In EAV-infected cells all of the three minor glycoproteins occur as a multimeric complex. However, only disulphide-linked Gp2/Gp4 dimers can be seen in infected cells. The trimer consisting of Gp2/Gp3/Gp4 is only detected in mature virions, in a time-dependent manner, which can be accelerated with alkaline pH and oxidative environment (Wieringa *et al.*, 2003b). This means that the disulphide linkage between Gp3 and the Gp2/4 dimer is catalysed outside the cell and not in the endoplasmic reticulum (ER). Deletion of either Gp2, Gp3 or Gp4 completely blocks the incorporation of the remaining two minor envelope glycoproteins, and reduces the amount of E in the virion (Wieringa *et al.*, 2004).

The minor glycoproteins are predicted as type I membrane proteins, with cleavable signal peptide (SP) and C-terminus located in the lumen of the ER. The membrane topology of the minor glycoproteins has so far been tested only with *in vitro*-synthetised proteins in the presence of canine microsomal membranes (de Vries *et al.*, 1995; Wieringa *et al.*, 2002). The 25 kDa Gp2 and 28 kDa Gp4 are integral membrane proteins of the predicted type I membrane topology, with a cleavable signal peptide and a C-terminal hydrophobic domain as transmembrane region. Recently we showed that transiently expressed Gp3 possesses an uncleaved signal peptide, which however does not anchor the protein (Matczuk *et al.*, 2013).

The 25kDa Gp2 and 28kDa Gp4 contain one and four potential N-glycosylation sites (NXT/S consensus sequence) respectively. Gp2 and Gp4 form a dimer in infected cells. When expressed transiently (with vaccinia system) in cells, Gp2 and Gp4 do not only form a heterodimer as in infected cells, but also Gp2-Gp2 and Gp4-Gp4 homodimers and other multimeric structures (Wieringa *et al.*, 2003b). In Gp2, the cysteines at amino acid positions 48 and 137 are linked with an intrachain disulphide bond, whereas the cysteine at amino acid position 102 makes the covalent bond with Gp4 (Wieringa *et al.*, 2003a). In the Gp4

sequence there are eight cysteine residues, four of which are located in the predicted signal peptide, three in the ectodomain and one in cytoplasmic tail. The intra- and interchain covalent linkages have not been investigated in Gp4.

The assembly of Arteriviruses occur on the internal membranes, presumably ER-Golgi intermediate compartment (ERGIC) (Wada *et al.*, 1995). However, when expressed alone, both Gp2 and Gp4 localise in the ER (Wieringa *et al.*, 2002). Only correctly folded, and in case of the multimeric complexes, oligomerised proteins can be trafficked from the ER to the next compartment. Proper folding in the ER depends on many co-translational (signal peptide cleavage, N-linked glycosylation) and post-translational (disulphide linkage formation) protein modifications, and is assessed by the chaperone quality control system. If the protein is not folded correctly, it will aggregate or become translocated back to cytosol for degradation (Braakman and Bulleid, 2011).

It is unclear how the membrane proteins of Arteriviruses are retained in the ER. The most known ER retention signal, C-terminal di-lysine motif (Teasdale and Jackson, 1996) are absent in Gp2 and Gp4. It could be possible that the lenght of the transmembrane domians (TMD) of minor proteins is crucial for their retention. Many ER and Golgi proteins exhibit shorter TMDs compared to the proteins targeted to the plasma membrane, which is linked to the thickness of the lipid bilayer of those compartments (Sharpe *et al.*, 2010).

The expression, folding and formation of heterologous complexes in Arteriviruses has not been fully investigated. In this study we focused on Gp2 and Gp4. We analysed N-linked glycosylation and membrane topology. To study the function of the Gp2/4 dimer in the Arterivirus entry we wanted to express the complex on the plasma membrane. This would allow us to perform binding and membrane fusion assays. Therefore, the TMDs and cytoplasmic tails of Gp2 and Gp4 were excahnged with the corresponding regions of Influenza A haemagglutinin. The chimeric proteins were able to form a covalently associated heterodimer Gp2/4, but were not targeted to the plasma membrane. Nevertheless, the same constructs were used to investigate the covalent bonds within Gp2/Gp4 dimer.

4.3 Materials and methods

4.3.1 Cells

Chinese hamster ovary cells (CHO-K1, ATCC CCL-61) were maintained in adherent culture in Dulbecco's Modified Eagle's Medium (DMEM, PAN, Germany) supplemented with 10 % fetal calf serum (Perbio, Germany). Baby hamster kidney cells (BHK-21, ATCC C13) were grown in DMEM mixed 1:1 with Leibovitz L-15 medium (PAN, Germany) supplemented with 5 % fetal calf serum (Perbio, Bonn, Germany). Cells were maintained at 37 °C in a humidified incubator with 5 % CO_2.

4.3.2 Plasmids

The full-length E (ORF2a), Gp2 (ORF 2b) and Gp4 (ORF 4) genes were PCR-amplified from the EAV expression vectors pAVI02a, pAVI02b and pMRI04 respectively (Snijder *et al.,* 1999; Weiland *et al.*, 2000; Wieringa *et al.*, 2002) and cloned between the *Xho*I and *Bam*HI sites of pEYFP-N1 or pECFP-N1 (Clontech), to encode minor EAV proteins C-terminally tagged with fluorescent protein (E-YFP, Gp2-YFP, Gp4-YFP and Gp2-CFP). The linker (amino acid sequence GMAPGRDPPVAT) between glycoprotein and fluorescent protein is derived from the vector's multiple cloning site. The nucleotide sequences encoding Gp2 and Gp4 with exchanged transmembrane and cytoplasmic tail of haemagglutinin (strain A/Fowl plague virus/Rostock/8/1934, GenBank: M24457.1) was *in vitro*-synthesised (Eurofins MWG Operon, Ebersberg, Germany). The Gp2 construct contained the linker LRPEAPRARDPPVAT and the HA tag sequence at the C-terminus (Fig. 4.1). In some experiments the Gp4 construct with C-terminal Flag-tag (DYKDDDDK) was used. The constructs were cloned into ACEMBL expression system *Multi*Mam (ATG:biosynthetics, Germany), Gp2 into the acceptor vector pACEM1 with *Xba*I and *Bam*HI restriction sites, and Gp4 into the donor vector pMDK with *Kpn*I and *Xho*I restriction sites. The acceptor and donor vectors were combined together with Cre-lox recombination (New England BioLabs, Germany). To abolish formation of disulphide linkages within the Gp2/Gp4 complex, the mutations C26S, C50S and C77S were introduced in the Gp4 sequence in pMDK with overlap extension PCR (Green and Sambrook, 2012). The plasmids were amplified in *E. coli* XL-1 blue (Stratagene/Agilent, Germany), except pMDK, which was amplified in the *pir* negative strain DH5α. Plasmids

were purified (PureYield Maxi Prep System, Promega,, Germany) and sequenced (GATC, Germany) before use in experiments.

4.3.3 Fluorescence protease protection assay

CHO-K1 cells seeded on 35-mm dishes with glass coverslips (MatTek Corporation, US) were transfected with TurboFect (Fermentas/Thermo) with plasmids pEYFP-E, pEYFP-Gp2 or pEYFP-Gp4. Twenty-four hours after transfection, the fluorescence protease protection assay was performed as described previously (Lorenz *et al.*, 2006). Briefly, digitonin (Sigma-Aldrich) concentration was adjusted with cells expressing YFP; it ranged between 20 µM and 40 µM. The digitonin was applied on cells for 60 s to permeabilase the plasma membrane, followed by addition of proteinase K (Sigma-Aldrich) at a final concentration of 50 µg/ml. Pictures were taken in 30-s intervals. Microscopy was carried out using an Olympus IX-81 epifluorescent microscope, pictures were processed using Image J software.

4.3.4 Metabolic labelling of proteins

CHO-K1 cells were seeded in 35-mm dishes and transfected with 4 µg plasmid DNA of pECFP-Gp2, and pEYFP-Gp4, using TurboFect (Fermentas/Thermo). Twenty-four hours after transfection, cells were starved for 2 h in medium lacking methionine and cysteine (Eagle's minimal essential medium with Earle's balanced salt solution, PAN Biotech, Germany). Subsequently, cells were labelled for 2 h with [^{35}S] protein labelling mix containing [^{35}S]methionine and [^{35}S]cysteine (5 µCi, MP Biomedicals, Germany) in methionine- and cysteine-deficient medium. After metabolic labelling, the medium was removed and the cells were lysed as described previously (Thaa *et al.*, 2009).

4.3.5 Immunoprecipitation and glycosidase treatment

The immunoprecipitation of YFP or CFP tagged constructs was performed with 2 µg of anti-green fluorescent protein (GFP) antibody (rabbit anti-GFP, A11122, Invitrogen) as described previously (Thaa *et al.*, 2009). The pelleted protein A-Sepharose with bound antigen-antibody complex was resuspended in 1× glycoprotein denaturing buffer (final concentrations: 0.5 % SDS, 40 mM DTT) and incubated at 100 °C for 10 min. For deglycosylation, aliquots of these denatured samples were digested with peptide-N-glycosidase (PNGase) F or endoglycosidase H (Endo H) according to the manufacturer's

instructions (New England BioLabs, Germany) for 1 h at 37 °C. For limited PNGase F digestion, a serial twofold dilution of PNGase F (starting with 1 unit/µL) was prepared for incubation with aliquots of the lysate in the same manner. The immunoprecipitation with the anti-Flag (anti-mouse, Sigma) was performed in MNT buffer (20 mM MES, 30 mM Tris, 100 mM NaCl, 1% TX-100, pH 7.4), antibody-protein complex was pulled with G-Sepharose (Sigma- Aldrich), washed with MNT and boiled with reducing or non-reducing SDS-buffer.

4.3.6 SDS-PAGE, Western blotting, fluorography

SDS-PAGE, Western blotting and fluorography were carried out as described previously (Matczuk *et al.*, 2013)

4.3.7 Immunofluorescence and confocal microscopy

Gp2/Gp4 with exchanged transmembrane regions and cytoplasmic tails was expressed in CHO-K1 cells on glass coverslips, and immunofluorescence was performed as described previously (Thaa et al., 2009). Rabbit anti-HA tag (1:2000, Invitrogen) and mouse anti-membrin (1:500, Abcam) were used as primary antibodies, and anti-rabbit Alexa 568 (1:2000) and anti-mouse Alexa 488 (1:2000) as secondary antibodies. Confocal microscopy was carried out on Olympus FV-1000 confocal laser-scanning microscope with an 60× objective and suitable lasers to sequentially excite the fluorophores.

4.3.8 Reverse genetics

To generate a mutant EAV carrying the C26S, C50S or C77S mutation in the Gp4 protein, following mutations were introduced into the EAV full-length cDNA clone pEAV211 (van den Born et al., 2005; van Dinten et al., 1997): a G to C mutation at nucleotides 10775 for C26S, G to C and T to C at nucleotides 10847 and 10848 for C50S, and G to C and T to C at nucleotides 10928 and 10929 for C77S. The mutation was first engineered in a *Bam*HI–*Eco*RI fragment (nt 9150–11488), which was sequenced (GATC Biotech) and transferred to pEAV211. Full length clones were linearised with *Xho*I and *in vitro*-transcribed using AmpliCap –Max T7 High Yeld Message Maker Kit (Biozym), and 5 mg RNA was then introduced into BHK-21 cells using Amaxa Nucleofector technology (Nucleofector kit L; program A-039), followed by incubation of the cells at 37 °C for 3 days. The immunofluorescence with anti-N monoclonal antibody (VMRD, USA) was

performed 16 h post-nucleoporation. Plaque assays were performed using Dulbecco's minimal essential medium containing 1 % FCS and 1 % glutamine, and stained with the neutral red after 3 days.

4.4 Results

4.4.1 Gp2-YFP and Gp4-YFP show type I membrane topology and localise to the ER

To analyse cellular localisation of Gp2 and Gp4, the proteins were fused to the yellow-fluorescent protein (YFP) at the C terminus. The resulting Gp2-YFP and Gp4-YFP were expressed in CHO-K1 cells (Fig. 4.1). The fluorescently tagged protein showed reticular distribution, resembling the ER. Membrane topology of Gp2-YFP and Gp4-YFP expressed in cells was confirmed with the fluorescent protein protection assay. The plasma membrane was permeabilised with digitonin, which acts on cholesterol and in low concentrations does not affect the internal membranes, followed by proteinase K treatment.

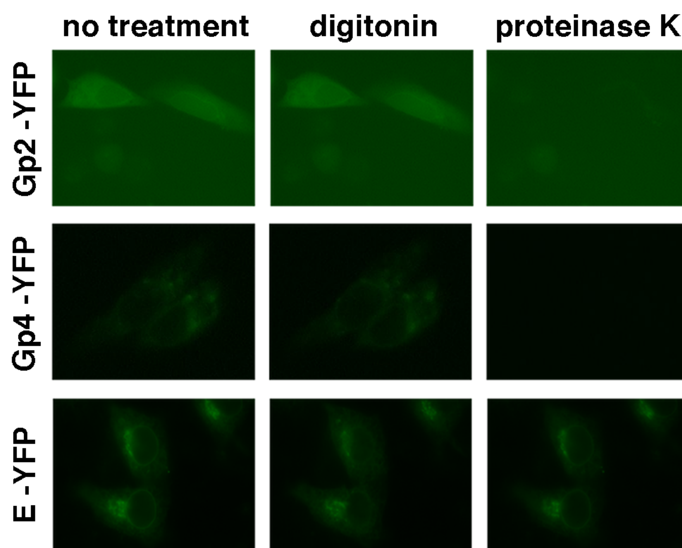

Figure 4.1 The C-terminus of Gp2 and Gp4 is not translocated into the lumen of the ER. CHO-K1 cells expressing Gp2-YFP, GP4-YFP or E-YFP as indicated were treated with digitonin (30 μM) for 1 minute and with Proteinase K (50 μg/ml) for 4 minutes. After each time point the same microscopic field was recorded with an epifluorescence microscope.

The YFP tagged to Gp2 and Gp4, and therefore the fluorescence signal, was not protected from proteinase K. This indicates that the C terminus of Gp2 and Gp4 was located in the cytoplasm. The E-YFP was used as an assay control. The E protein is predicted as type II membrane protein, and its topology was experimentally proven in PRRSV E (Yu *et al.*, 2010). In contrast to Gp2-YFP and Gp4-YFP, in the E-YFP protein, the YFP at the C terminus was protected from proteinase K digestion, meaning that it is oriented toward the lumen of cellular internal membranes.

4.4.2 Gp2-CFP and Gp4-YFP do not pass the medial Golgi compartment

To study the fate of the minor glycoproteins when expressed in cells, Gp2-CFP and Gp4-YFP expressed in CHO-K1 cells were metabolically labelled with [^{35}S]cysteine and [^{35}S]methionine, immunoprecipitated with anti-GFP antibody and analyzed by SDS-PAGE and fluorography. Upon deglycosylation with PNGase F or endoglycosidase H (Endo H) the size of both proteins decreased (Fig. 4.2A).

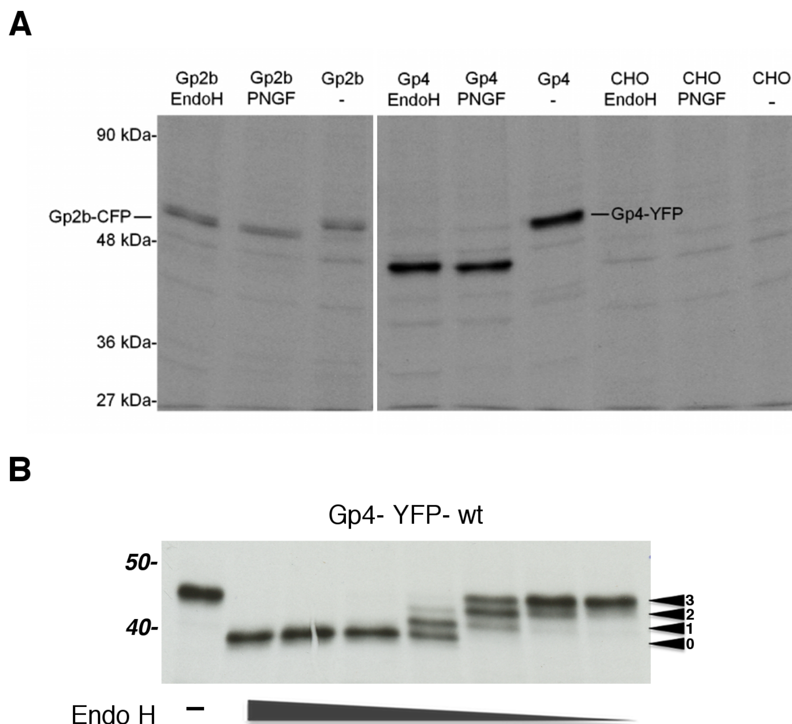

Figure 4.2 Gp2-CFP and Gp4-YFP do not pass medial Golgi compartment. Gp4-YFP contains three N-linked glycans. (A) Gp2-CFP and Gp4-YFP were labelled for 2 h with [^{35}S]-methionine/cysteine in transfected CHO cells, immunoprecipitated and digested with

Endo H or PNGase F as indicated prior to SDS-PAGE and fluorography. **(B)** Gp4-YFP, labelled for 2 h with [^{35}S]-methionine/cysteine in transfected CHO cells, was immunoprecipitated with anti-GFP antibodies and digested with serial twofold dilutions of Endo H prior to SDS-PAGE and fluorography. The numbered arrows indicate the number of carbohydrates remaining on the protein bands.

PNGase F cleaves off N-linked oligosaccharide side chains from proteins irrespective of their maturation state, while Endo H is only able to cleave off high mannose carbohydrates. Gp2-CFP and Gp4-YFP were sensitive to Endo H, meaning that they did not pass the medial Golgi compartment where Endo H resistance is acquired. The size shift was small in case of Gp2-CFP, because it only has one N-glycosylation sequon. In order to count the number of the carbohydrate residues in Gp4-YFP, limited digestion with serially diluted Endo H was performed (Fig.4.2B). Gp4-YFP possessed three sugar moieties, an expectable result as one of the four predicted sequons has proline just after the asparagine residue.

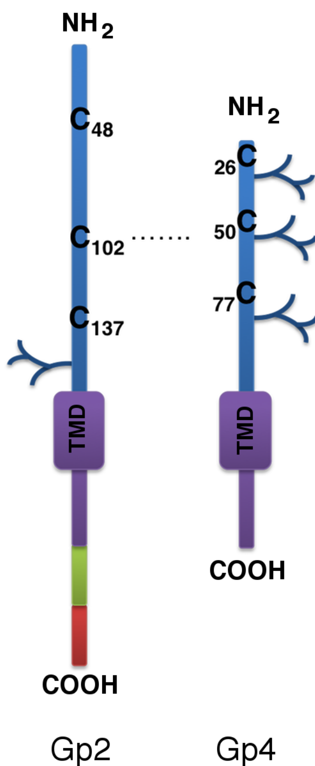

Figure 4.3 Schematic picture of the Gp2 and Gp4 constructs used in the study. The cysteines with amino acid positions are marked. Dotted line from C102 indicates known disulphide bond. Blue: ectodomain of EAV minor glycoprotein, violet: TMD and cytoplasmic tail of HA, green: linker, red: HA tag, branch: N-linked glycan.

4.4.3 The exchange of TMD and cytoplasmic tails of Gp2 and Gp4 does not target proteins to plasma membrane

The retention signals of EAV membrane proteins are unknown. We hypothesized that exchange of the transmembrane domains (TMD) and the cytoplasmic tails by that of a known plasma membrane protein would allow expression on the cell surface, and therefore facilitate functional studies of the Arterivirus minor glycoproteins. In order to express Gp2/4 on the cell surface we exchanged putative TMDs and cytoplasmic tails of both proteins with the corresponding regions of influenza A virus haemagglutinin, since this is a well characterised protein efficiently transported to cell surface. Because earlier we tested the topology, we can be sure that topology of Gp2 and Gp4 is the same as haemagglutinin. The Gp2-HA-TMD protein was tagged with an HA tag for detection by immunofluorescence, while the Gp4-HA-TMD remained untagged (Fig. 4.3). Both proteins were co-expressed in mammalian cells using the multi-expression vector system containing two separates promoters. The protein expressed from this construct was named Gp2-HA/Gp4. Under reducing conditions in SDS-PAGE and Western blot with anti-HA tag, we observed a 24-kDa band, which corresponds to the Gp2-HA-TMD-HA tag protein. Under non-reducing conditions, a 50-kDa band was detected (Fig. 4.4A). To prove that this corresponds to a heterodimer composed of the Gp2 and Gp4 probes, the metabolically labelled glycoproteins were co-immunoprecipitated under non-denaturating conditions with anti-HA tag antibody (Fig. 4.4B). The predicted sizes of Gp2-HA-TMD-HA tag and Gp4-HA-TMD are very similar, with molecular weight of 26 kDa and 25 kDa respectively. This explains why the bands co-migrated in the SDS-PAGE, as a reducing buffer was employed for deglycosylation (DTT-containing deglycosylation buffer). As the Gp4-HA-TMD has three N-linked glycans, the backbone of the protein differs significantly from Gp2-HA-TMD-HA. Further deglycosylation of these samples with either PNGase F or Endo revealed that indeed the dimers are composed of Gp2 and Gp4 (Fig. 4.4B). All the N-glycans from the immunoprecipitated Gp2 and Gp4 probes remained Endo H-sensitive, which suggests that the proteins did not pass the medial Golgi compartment.

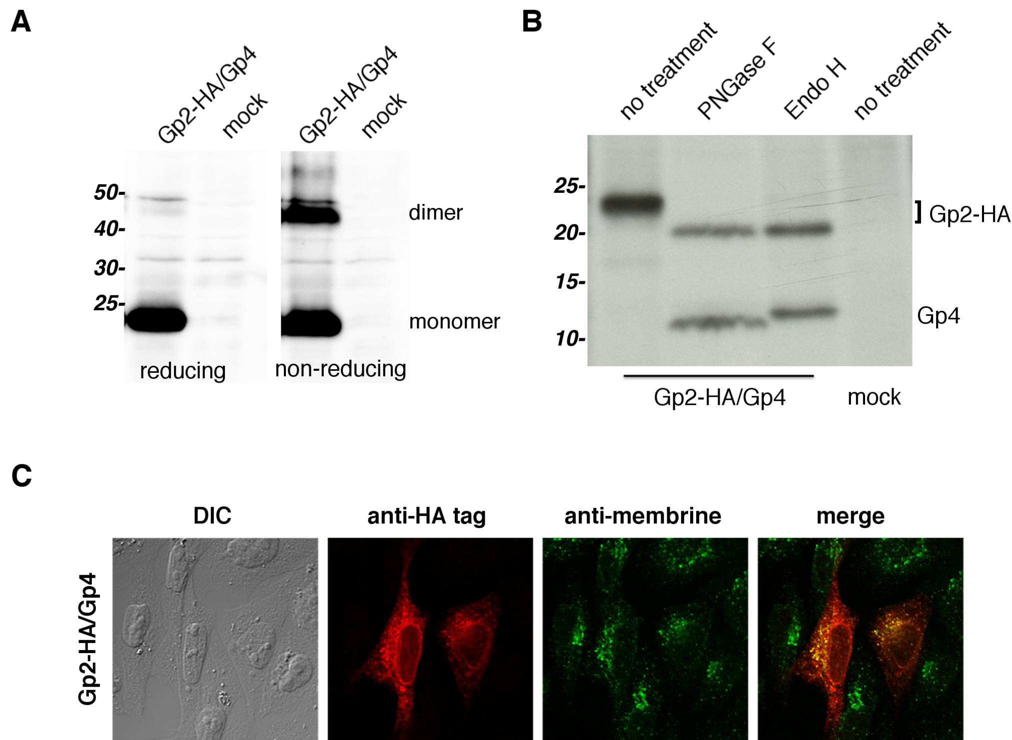

Figure 4.4 Dimerisation and subcellular localisation of Gp2 and Gp4 dimers with exchanged TMD and cytoplasmic tails. (A) Gp2-HA/Gp4 was expressed in CHO cells and detected by Western blotting with the anti-HA antibodies after cell lysis in reducing and non-reducing buffer. Molecular weight markers indicated next to gel pictures. *mock*: sample from untransfected cells. **(B)** Gp2-HA/Gp4, labelled for 2 h with [^{35}S]-methionine/cysteine in transfected CHO cells, was immunoprecipitated with anti-HA antibodies and digested with PNGase F or Endo H prior to SDS-PAGE and fluorography. **(C)** Gp2-HA/Gp4 was expressed in CHO cells seeded on coverslips. Immunofluorescence was performed with anti-HA and anti-membrin antibodies (cis-Golgi marker). The merge picture was generated with the Image J software.

Immunofluorescence studies with anti-HA antibodies showed reticular staining and only partial co-localisation with the cis-Golgi, stained with an antibody against the marker protein membrin (Fig. 4.4C). No plasma membrane expression was detectable, neither with fluorescence microscopy nor with a surface biotinylation assay (data not shown). To summarize, the co-expression of Gp2-HA-TMD-HA tag and Gp4-HA-TMD enabled these

two proteins to form a heterologous dimer and partial trafficking from the ER to the cis-Golgi.

4.4.4 Gp4 with single mutated cysteine residues is still able to form dimers with Gp2

As we were capable to show the presence of Gp2-Gp4 dimers in our chimeric constructs, we tried to analyze which cysteine in Gp4 is responsible for disulphide bond formation to the Gp2. The three cysteines (C) located in the Gp4 ectodomain, C26, C50 and C77, were mutated individually (Fig. 4.3). It was shown with Gp4 expressed in insect cells that the signal peptide is cleaved between amino acid 21 and 22 (Sinhadri Balaji, FU, Berlin, unpublished data), thus we ruled out that the cysteine residues in the signal peptide could be engaged in the covalent linkage with other minor glycoproteins. The result from the membrane topology study (Fig. 4.1) indicates that the cysteines in the cytoplasmic tail of Gp4 are not located in the lumen of the ER, thus also cannot form intra or inter-chain disulphide linkages in the complex. To facilitate the analysis of dimer composition, a Flag tag was C-terminally added to the Gp4 construct.

The two proteins were expressed together in the same manner as mentioned earlier. In Western blot with anti-HA antibody we detected the Gp2, which under non-reducing condition formed dimers in all Gp4 mutants (Fig. 4.5A). By Western blot with anti-Flag tag antibody, dimer formation was observed for wild type Gp4, Gp4 C26S, and Gp4 C50S, but not in Gp4 C77S (Fig. 4.5B). It was previously shown that in transient expression of minor proteins, not only Gp2/Gp4 heterodimers, but also homodimers of Gp2 and Gp4 are formed (Wieringa *et al.,* 2003b). To investigate if the dimers we observed are indeed the heterodimers, we subjected cell lysates of transfected cells to immunoprecipitation (IP) with anti-Flag tag antibody in condition preserving the complex. Following IP, the samples were boiled in non-reducing SDS-PAGE buffer, and proteins were electrophoresed and visualized with Western blot using antibodies against HA tag and Flag tag. Upon pull-down of Gp4, both Gp2 (anti-HA) (Fig.4.5C) and Gp4 (anti-Flag) could be detected (Fig. 4.5D). This result indicates that the dimers were indeed composed of Gp2-HA-TMD- HA and Gp4-HA-TMD-Flag. However, deletion of just one cysteine in the Gp4-HA-TMD-Flag ectodomain did not abolish formation of the dimers. In the C77S mutant, the dimer band was not visible in total cell lysate control, but existed in the enriched IP sample.

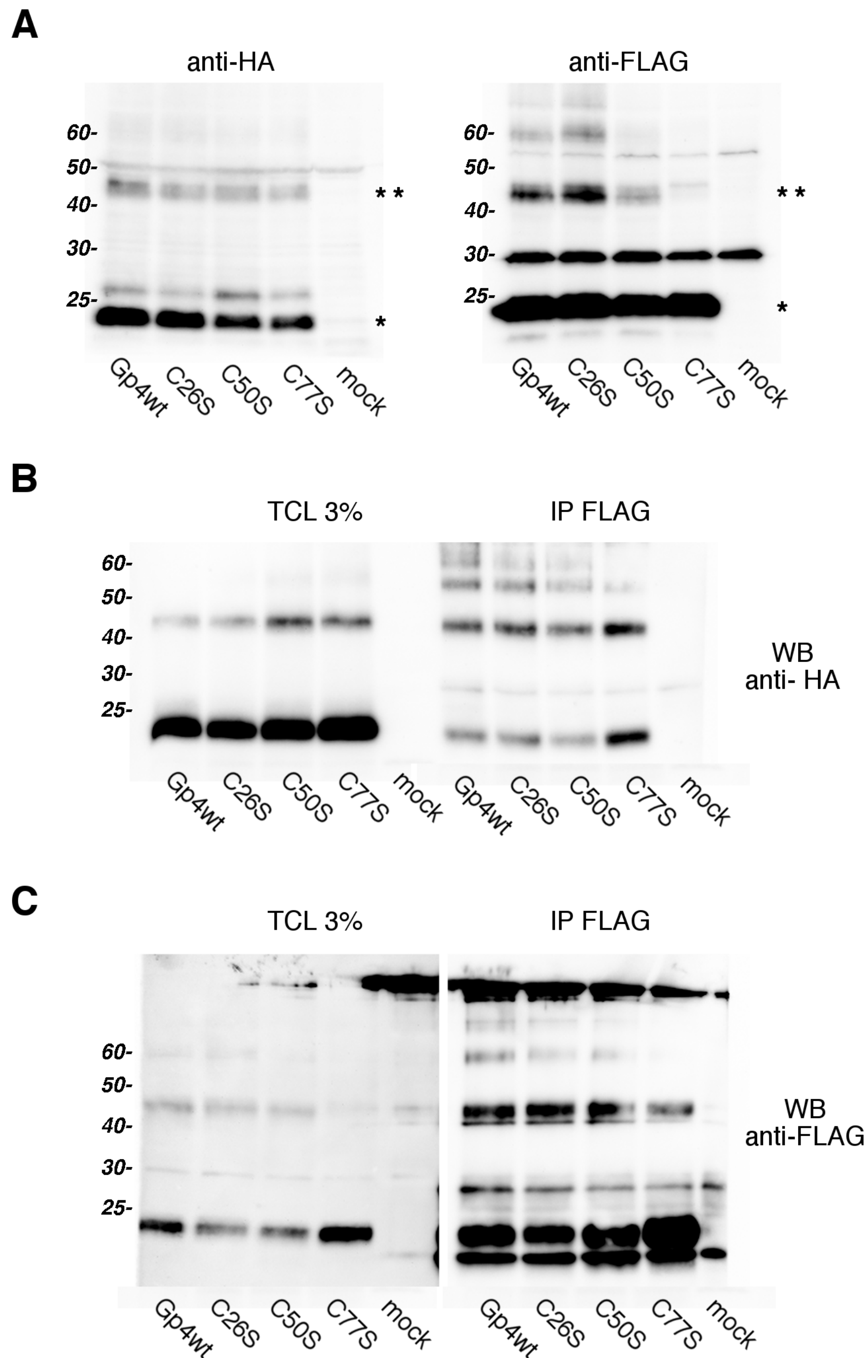

Figure 4.5 The single cysteine mutations in Gp4 do not influence Gp2-HA/Gp4-Flag dimerization. (A) Gp2-HA/Gp4-Flag wild type and constructs with cysteine mutations in Gp4 were expressed in BHK cells, lysed in non-reducing SDS-PAGE buffer and detected by Western blotting with anti-HA tag or anti-Flag tag antibodies. Monomers (*) and dimers

(**) are indicated with stars. **(B)+(C)** Gp2-HA/Gp4-Flag wild type and constructs with cysteine mutations in Gp4 were expressed in BHK cells and lysed in MNT buffer. 6% of lysate was boiled with non-reducing SDS-PAGE buffer, and the remaining lysate was subjected to IP with anti-Flag tag antibody. Proteins were detected by Western blot using anti-HA tag **(B)** or anti-Flag tag **(C)** antibodies. Molecular weight markers indicated next to gel pictures. IP: immunoprecipitation, WB: Western blot, TCL: total cell lysate, *mock*: sample from untransfected cells.

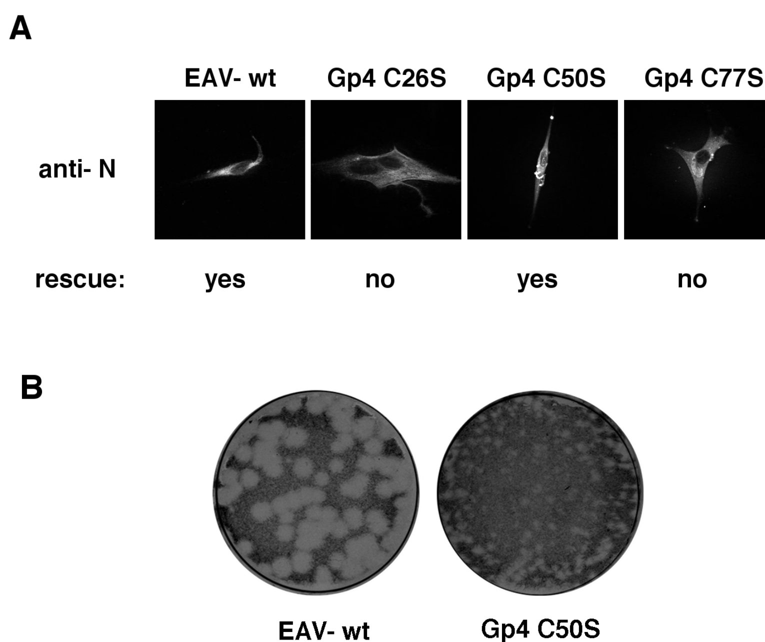

Figure 4.6 Gp4 cysteine mutants virus rescue and plaque morphology. (A) BHK cells were nucleoporated with mRNA reverse-transcribed from pEAV211 wt or carrying the indicated mutations in the Gp4-encoding gene. Immunofluorescence with anti-N antibody was performed 16 h post-nucleoporation. **(B)** Representative plaque assay of EAV wt and Gp4 C50S mutant.

4.4.5 The cysteine residues in the ectodomain of EAV-Gp4 are essential for virus infectivity

To study the role of luminal cysteine residues of Gp4 in complex formation with the Gp2 and Gp3, as well as for their significance in virus infectivity, the Gp4 mutations C26S,

C50S and C77S were introduced individually into the full-length EAV cDNA clone for generation of recombinant viruses. The efficiency of nucleofection of the mRNA was checked with immunofluorescence using an antibody against the nucleocapsid protein (Fig. 4.6A), for all mutants as well as wild type, we observed expression of N, meaning that nucleoporation of cells was successful with each mRNA. However, the rescue performed three times yielded progeny virus only in case of wild type and the mutant Gp4 C50S. The supernatants were incubated with fresh cells to propagate the virus, or directly used in plaque test. The RNA from those cells was reverse-transcribed and PCR-amplified to confirm the rescue. No virus was obtained when Gp4 was mutated at positions C26 and C77. The rescue of virus carrying the Gp4 C50S mutation was possible, but its propagation was not effective, as the highest titre obtained was only 10^2 plaque-forming units (pfu)/ml, while in the same conditions the wild type grew to the titres of 10^7 pfu/ml. The virus mutant with Gp4 C50S formed much smaller plaques than the wild type virus (Fig. 4.6B).

4.5 Discussion

In this study we first investigated the membrane topologyof EAV proteins tagged with fluorophore in transfected cells. The membrane topology of Gp2-YFP and Gp4-YFP was type I (N-terminus in the ER lumen, C-terminus in the cytoplasm) which is consistent with studies performed with *in vitro* expressed proteins (Wieringa *et al.*, 2002). E-YFP has a different topology, its C-terminus was located in the lumen of ER. The topology of EAV-E has never been investigated, but the same topology was observed for the E protein of closely related PRRSV (Yu *et al.*, 2010). Even without colocalisation studies of with organelle markers, the expression pattern shows reticular appearance, typical for ER distribution, which was however not further backed by colocalisation analysis with organelle markers. The result implies that the minor proteins expressed separately are retained in the ER.

The related Coronaviruses, assemble by budding into membranes of the ERGIC. In prototype mouse hepatitis virus, the spike protein S, when expressed alone, localises to the ER or the plasma membrane. However, oligomerisation of S with the M protein already in the ER leads to retention of both proteins in the cis-Golgi, where M localises also when expressed alone (Opstelten *et al.*, 1995). This could indicate that the minor glycoproteins of EAV must oligomerise in order to colocalise in the site of budding, but it was never investigated, mostly because of the reverse genetic system limitation (nested genome).

In our study we observed formation of dimers composed of Gp2-HA-TMD-HA and Gp4-HA-TMD, those complexes localised to the cis-Golgi, meaning that the dimers passed the ER quality control system and were exported to the next compartment. However, most of the Gp2-HA-TMD-HA protein was localised in the ER. Also in Western blots under non-reducing conditions, the monomeric forms of both Gp2-HA-TMD-HA and Gp4-HA-TMD-Flag were detected in high amounts. This indicates that these constructs were able to form the heterodimers, but that this process was not very efficient. This may be attributed to the introduced modifications of the TMD and cytoplasmic tail, or could be significant because Gp2 and Gp4 are highly expressed during infection but only small portions of them end up in the virion (de Vries *et al.*, 1995; Wieringa *et al.*, 2002). The most important finding in this study is that the ectodomains of Gp2 and Gp4 are sufficient for heterodimer formation.

Since we demonstrated the heterodimer formation, we wanted to investigate which cysteine in the Gp4 is responsible for dimerisation with Gp2. In our system, where we expressed only Gp2 and Gp4 (as chimeras) the single mutations of cysteines in Gp4 did not abolish formation of heterodimers. This could indicate that even if one cysteine in Gp4 is mutated the other two could still compensate and form the dimer with Gp2.

In the virion, Gp2/4 is covalently linked to Gp3. While no covalent association of Gp3 with Gp2/4 is observed in cells, we thought that the absence of Gp3 in our system could affect the folding and disulphide linkage formation between Gp2 and Gp4. To overcome lack of Gp3 and check the impact of cysteine mutations in Gp4 on EAV infectivity, we mutated the individual cysteines in Gp4 in the virus context. In Gp4, one of the cysteines has to form a linkage to the essential C102 in Gp2, and another cysteine the bond to Gp3. We could not rescue mutant viruses with C26 and C77 deletions and the C50 mutant was severly affected in growth. This is surprising, because according to previously postulated trimer model (Wieringa *et al.,* 2003a), there is only one disulphide linkage between each of minor glycoprotein. Therefore, we expected that one out of three cysteines will be dispansable. Due to the low titre of Gp4 C50S mutant, we could not analyse the trimer formation of this mutant, therefore we do not know which cysteine in Gp4 is responsible for association with Gp3. Further experiments are needed to reveal the nature of covalent linkages between the minor envelope proteins.

From our data, we can conclude that Gp4 might form a dimer with Gp2 even if one of the Gp4's cysteine is deleted. Therefore the dimer formation is quite flexible in

transiently expressed proteins. However, for the protein funcion, which is virus entry, it is crucial to have all cysteins in the Gp4 ectodomain present. During the folding process, the disulphide bonds can be broken and the new one can be formed to achieve the native Gp2/4 complex. In the virus context, in the Gp4 cystein mutants, this is probably not fulfiled. Our experiments were conducted with chimeric constructs, where folding and oligomerisation proprieties of Gp2 and Gp4 might be changed. However, the complex was transported to the cis-Golgi, which means that it passed the ER quality system. It would be optimal to analyse dimerisation of the native, unchanged Gp2 and Gp4, which was not possible here due to lack of good antibodies.

As we demonstrated that the TMD of minor glycoproteins could be exchanged and the heterodimers were still formed, this knowledge can be used for future investigations, such as creation of the pseudoviruses. In such artificially ingenered viruses the ectodomains could be substituted with ectodomains of Gp2 and Gp4, which could facilitate the funcional studies on those proteins.

4.6 References

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Chapter 5

General discussion

Two members of the *Arteriviridae*, EAV and PRRSV, are important pathogens of domestic animals. The former causes problems in horse reproduction and restrictions in semen shipment, while the latter causes extremely high economic losses in swine industry worldwide. The research on *Arteriviridae* biology focuses mainly on virus replication and to a certain extent on pathogenesis and humoral immune responses (Snijder *et al.*, 2013). At the same time, the experimental data on the minor envelope glycoproteins, which were shown to be essential for virus tropism, are very limited (Tian *et al.*, 2012).

In this dissertation, I describe the unique co-translational processing of Gp3 where the addition of N-linked glycans adjacent to signal peptide (SP) cleavage site prevents the cleavage. I describe the membrane topology of Gp3, where uncleaved SP does not act as a signal anchor. I also focus on the topology of Gp2 and Gp4, their dimerization and effects of cysteine mutation on virus infectivity.

5.1 Membrane topology of Gp3

The Gp3 protein of EAV is a part of the Gp2/3/4 minor glycoprotein complex. It associates covalently (with disulphide bond) with the Gp2/4 dimer only after release of virions (Wieringa *et al.*, 2003b). The membrane topology of Gp3 has not been resolved for any of the Arteriviruses. For viral structural proteins, the membrane topology will define which parts of the protein will be hidden inside the virion, and which parts will protrude from its surface. The Gp3 of EAV is predicted to be a type I membrane protein, with cleavable SP and C-terminal hydrophobic domain as a transmembrane domain (TMD) (Fig. 1.4B-c). However, previous data from *in vitro* transcription-translation studies with the use of canine microsomal membranes showed that the SP is not cleaved and that the protein is only luminal (Wieringa *et al.*, 2002). This led to the assumption that the uncleaved signal peptide may act as a membrane anchor, and two topology models were proposed (Wieringa *et al.*, 2003a), where Gp3 is either anchored with its N-terminus only, or via both hydrophobic termini: N- and C- (Fig.1.4B a and b).

My topology experiments with fluorescent protein protease protection assay (Fig. 2.5) showed that in Gp3 the C-terminus is located in the ER lumen, suggesting that the C-terminal hydrophobic region is fully translocated and does not form a TMD. Additionally,

as Gp3 is an integral membrane protein, the membrane topology model, consistent with these data, would be type II (Fig. 1.4B–a), where the uncleaved SP anchors the protein in the lipid bilayer, and the C-terminus is located in the ER lumen.

In Gp3 the very reliable signal peptide prediction software SignalP4.0 calculates a high probability of cleavage between amino acids 26 and 27 (Fig. 1.6C). However, I confirmed that indeed the SP of Gp3 is uncleaved. The protein synthetized *in vitro*, in cell free system characterised by lack of glycosylation and SP cleavage, ran identically in SDS-PAGE as degycosylated Gp3 obtained from transfected cells (Fig. 2.2A). The contradictory results in software predictions and experimental data may be explained by the fact that such predictions do not take into consideration co- and post-translational modifications of the protein such as glycosylation. Therefore, I hypothesised that glycans on asparagines at positions 28 and 29 might interfere with SP cleavage. Indeed, in Gp3 lacking N-glycans adjacent to the SP cleavage site, the SP was cleaved off while in wt Gp3 it was retained (Fig. 2.3A–C) (See also 5.3).

As I demonstrated that the SP is not cleaved off in Gp3 synthetized in transfected cells, I asked if it could be responsible for membrane anchoring. The membrane association experiments showed that with or without SP, the Gp3 is always membrane-bound (Fig. 2.5). However, deletion of the hydrophobic C-terminus allowed secretion of Gp3 into the supernatant regardless of the presence or absence of SP, indicating that only the C-terminal hydrophobic domain, and not the SP, is responsible for membrane anchoring of Gp3 of EAV (Fig. 2.6A). Therefore, it appears likely that during Gp3 synthesis the SP slips out of the Sec 61 channel during translocation (Fig. 5.2B). Such an event has been observed before. For instance, the SP of the p62 protein of Semliki Forest Virus (SFV) is not cleaved off, but translocated and quickly glycosylated (Garoff *et al.*, 1990). Another, non-viral example is corticotropin-releasing factor receptor type 2A. The non-anchoring, uncleaved SP, called by the authors “pseudo signal peptide” prevents oligomerisation of this receptor (Teichmann *et al.*, 2012).

One can imagine, that the SP slips into the ER, because the interaction of SP with the luminal part of translocation channel is suboptimal. It is possible that the uncleaved signal peptides of SFV p62, corticotropin-releasing factor receptor type 2A and Gp3 share undefined features allowing them to leave the translocon channel not through integration within the lipid bilayer, but rather to the ER lumen.

5.2 The hydrophobic C-terminus is responsible for membrane association of Gp3

As mentioned already, the C-terminal HR is responsible for membrane anchoring of Gp3. Because the tag inserted at the C-terminus of Gp3 was translocated into the ER lumen it is unlikely that this HR is the TMD of Gp3 spanning the ER membrane completely. However, TMDs are not the only way to achieve membrane anchoring of protein. Amphipathic helices are alpha helices that have two distinct faces with different properties. One surface of the amphipathic helix contains hydrophilic amino acids, which prefer the aqueous surrounding, while the opposite surface has hydrophobic amino acids, which favor lipid environment. I hypothesized that the C-terminal HR of Gp3 may be an amphipathic helix, therefore I performed a helical wheel plot. As depicted in Figure 5.1, the HeliQuest software was used to predict the helix property of the hydrophobic C-terminus of the Gp3. The analysed region has a large hydrophobic part interspersed with polar amino acids: arginine, histidine, threonine and proline, a helix breaker. The helix lacks distinct hydrophobic and hydrophilic faces; therefore it is unlikely that it tethers the Gp3 peripherally to the membrane. More likely the hydrophobic C-terminus is embedded in the ER membrane, but does not span it completely.

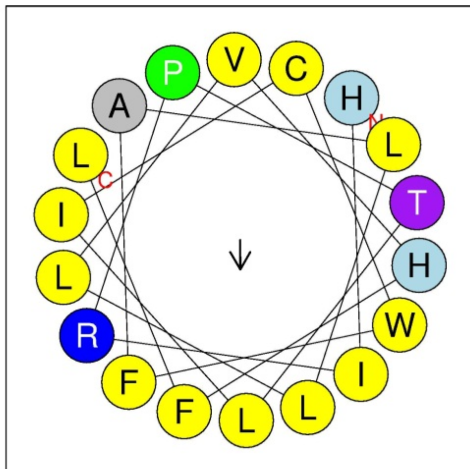

Figure 5.1 Helical wheel plot analysis of the C-terminal hydrophobic part of EAV Gp3. The amino acid sequence 138–155: HIRPTLICWFALLLVHFL was subjected to analysis with HeliQuest software (http://heliquest.ipmc.cnrs.fr). Hydrophobic face (yellow and gray) broken with arginine residue (blue). Helix breaker proline shown in green. Histidine shown in light blue and threonine in violet.

Such a hairpin topology was shown for E protein from avian infectious bronchitis virus (IBV) (Ruch and Machamer, 2012), and was dependent on the presence of a single polar residue – threonine located in the N-terminal part of hydrophobic domain. The C-terminal HR of Gp3 stretches from amino acid residues 137 to 155. Alignment of 249

sequences of EAV Gp3 from GenBank revealed that this domain is not highly conserved, with the exception of arginine on amino acid position 140, which is present in every Gp3 sequence (not shown). Threonine and arginine have different size, but they are both polar amino acids. It may be possible that threonine and arginine residue located at the N-terminal end of the C-terminal hydrophobic region of E from IBV and Gp3 of EAV respectively, contribute to the unusual topology of those proteins. The reason why the HR of Gp3 adopts the hairpin-like structure needs further investigation.

Another example for a transmembrane domain not spanning the membrane completely is the E1/E2 envelope protein of hepatitis C virus (HCV). When expressed alone E1 and E2 exhibit hairpin like topology with both termini located towards the lumen of the ER. However when expressed together or as a viral polyprotein, the topology changes. After cleavage by SPase the C-terminal ends of E1 and E2 flip back across the membrane converting the hairpin into a transmembrane helix (Cocquerel *et al.*, 2002).

My topology experiments were performed with transfected cells with the Gp3 protein tagged at the C-terminus with YFP. This studies, together with the data describing membrane anchoring, allowed me to postulate the new topology model of the Gp3 of the EAV, where N- and C- termini are located in the lumen of ER, the uncleaved SP is not a membrane anchor and the hydrophobic C-domain has hairpirin structure embedded in the ER membrane (Fig 2.7A). As the hydrophobic hairpin structure of E1 and E2 glycoproteins of HCV can flip across the membrane after the proteins' translocation to ER due to the presence of partner protein, it also might be possible for other TMDs. Therefore, it could be possible that the C-terminal HR of the Gp3 is membrane-spanning in the virus, because there are other viral components such as Gp2/Gp4 and E during infection. However, due to nested genome of the EAV, it is not possible to insert the tags into Gp3 without disturbing the ORF4. The future studies are needed to confirm the topology model of the Gp3 in the virion, or at least when other minor proteins are co-expressed. Also the properties of the C-terminal HR should be studied in detail, e.g. what role plays conserved arginine on position 140.

EAV is the prototype Arterivirus. Does the topology model of Gp3 apply for all members of this family? Gp3 varies in length and hydrophobicity among Arteriviruses. The length and hydrophobicity plot of EAV Gp3 (Fig. 1.4A) resembles that of SHFV Gp3 (Fig. 5.2), whereas the Gp3 of LDV and especially of both European and North American

genotypes of PRRSV have longer amino acid sequences and their C-terminal part is much less hydrophobic (Fig. 5.2). Lack of hydrophobicity in the C-terminus is especially visible in the PRRSV European genotype, which is depicted with an arrow in the figure 5.2.

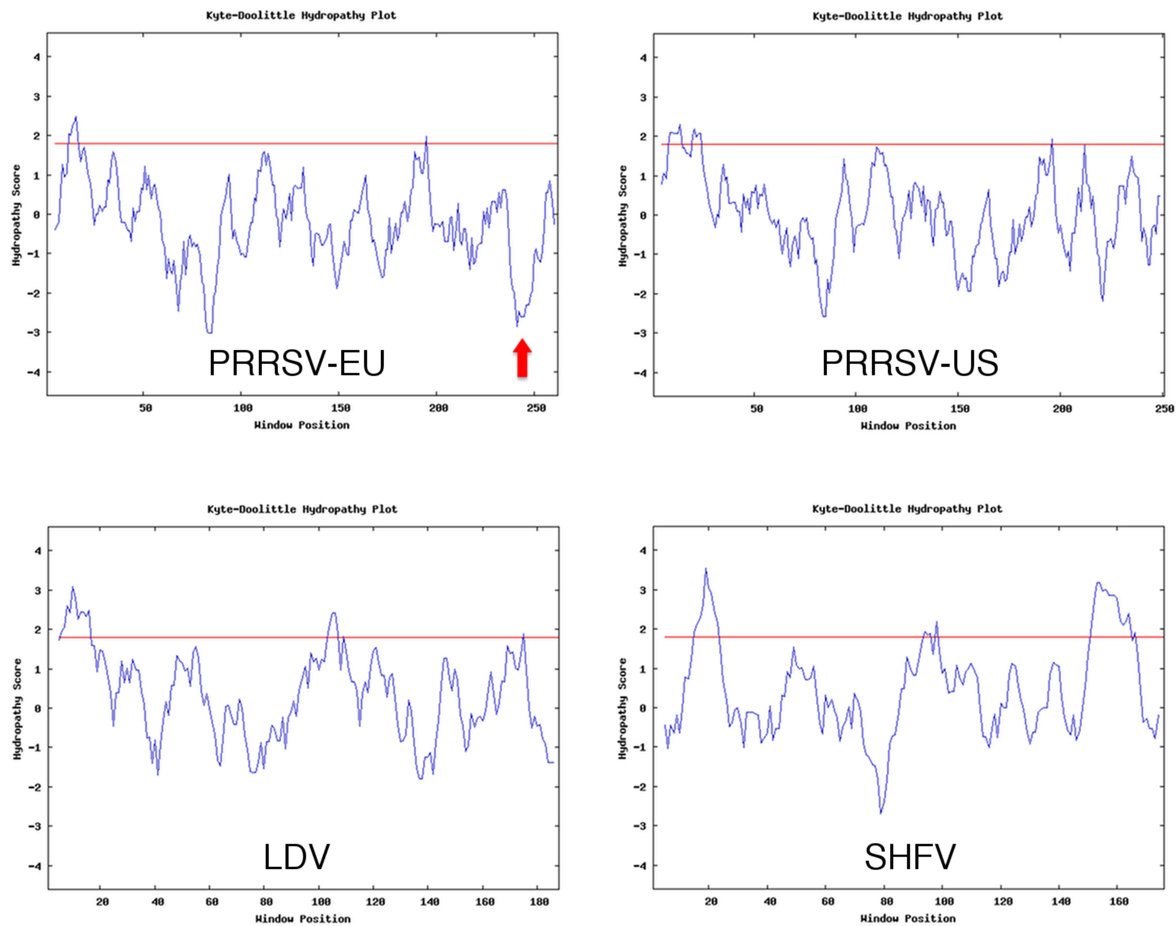

Figure 5.2. Kyte-Doolittle hydrophaty plot of Gp3 from all *Arteriviridae* family members. y-axis: hydropathy score, x-axis: amino acid position. Red line indicates a threshold of transmembrane domain prediction (http://gcat.davidson.edu/DGPB/kd/kyte-doolittle.htm). PRRSV-EU: Porcine reproductive and respiratory syndrome virus, European genotype (strain Lelystad); PRRSV-US: Porcine reproductive and respiratory syndrome virus North American genotype (strain VR-2332); LDV: lactate dehydrogenase-elevating virus Plagemann strain. SHFV: Simian haemorrhagic fever virus. Red arrow indicates the main difference in hydrophobicity of C-terminus between PRRSV-EU and PRRSV-US.

In LDV, the Gp3 was described as a secreted and not virion-associated protein (Faaberg and Plagemann, 1997). In contrast, the Gp3 of European genotype PRRSV is structural viral protein (van Nieuwstadt *et al.*, 1996). For the North American genotype of PRRSV, Gp3 was first described as a secreted, non-structural protein (Mardassi *et al.*, 1998), later studies showed that Gp3 is a structural protein (de Lima *et al.*, 2009). The discrepancies between these studies on the nature of Gp3 in PRRSV North American genotype might be due to different virus strains and anti-Gp3 antibodies used in the experiments.

Virus strain	**Aa length**	**HR in C-terminus**	**Secreted**
EAV	163	yes	no
SHFV	182	yes	?
PRRSV-EU	265	no, very hydrophilic C- terminus	no
PRRSV-US	254	no	yes/no
LDV	191	no	yes

Table 5.1 The comparison of the Gp3 in *Arteriviridae*. aa: amino acid; HR: hydrophobic region; EAV: equine arteritis virus; SHFV: Simian haemorrhagic fever virus; PRRSV-EU: Porcine reproductive and respiratory syndrome virus, European genotype (strain Lelystad); PRRSV-US: Porcine reproductive and respiratory syndrome virus North American genotype (strain VR-2332); LDV: lactate dehydrogenase-elevating virus (Plagemann strain).

To summarize, the nature of the Gp3 protein appears to vary among *Arteriviridae,* which is combined in the Table 5.1. In EAV this protein was always reported as structural virion protein, while in LDV the Gp3 is secreted. In PRRSV both virion-associated and secreted Gp3 was observed. Thus, Gp3 may exist in two forms in PRRSV. If Gp3 proteins of all Arteriviruses have the same topology as in EAV, a dual nature of Gp3 might be due to the “strength” of the C-terminal anchoring to the membrane of the virion. If the hydrophobic C-terminus cannot associate the protein to the membrane, Gp3 would only be a secreted protein like in LDV. In PRRSV, especially in the North American genotype, Gp3 seems to

have a dual nature, indicating that the C-terminus has weak anchoring activity. One can imagine that it would be beneficial for the virus to possess some fraction of the structural protein also in a secreted version. Such a dual nature of the structural protein has been observed for glycoprotein D of herpes simplex virus type 2 and the E^{rns} glycoprotein of bovine viral diarrhea virus, the secreted versions of those proteins have chemokine binding activity or block interferon signaling, respectively (Iqbal *et al.*, 2004; Murata *et al.*, 2002).

Gp3 of PRRSV North American genotype contains neutralizing epitopes, but generally in PRRS the neutralizing antibodies are produced late during infection (Vu *et al.*, 2011). If the secreted Gp3 can "catch" those antibodies, less of them would be available for neutralization of the virions. Therefore, hypothetically such secreted Gp3 could act as a decoy protein. Another possibility is that the conformations of trimer-associated Gp3 in the virion could be different than that of secreted Gp3. Different conformation could result in new protein function or could stimulate distinct immunological response of the host, e.g. the antibodies against non-neutralising epitopes may be produced earlier in the infection.

5.3 N-linked glycans adjacent to the signal peptide cleavage site in Gp3 interfere with cleavage

The inconsistence of membrane topology predictions with previously published experimental data, which suggested uncleaved SP of Gp3, led us to hypothesise that the glycans adjacent to the SP cleavage site might interfere with SP cleavage.

Indeed, upon deletion of both glycosylation sites in the overlapping sequon the SP was cleaved, while wt Gp3 or Gp3 with only one site from the overlapping sequon deleted maintained the SP in the mature protein (Fig. 2.2). The glycosylating complex OST and the SPase complex are both associated with the translocation channel. When the inhibitor of N-linked glycosylation tunicamycin was used, the SP was cleaved also in wt Gp3, showing that the Gp3 has a functional cleavage site and that addition of N-glycans adjacent to cleavage site affects the cleavage (Fig.2.3B). In pulse chase analysis, I observed that all glycosylation sequons of Gp3 are modified co-translationally (Fig.2.3A). The observation that the N-linked glycans are added during translocation speaks in favor that during Gp3 synthesis the N-linked glycans are added before the SPase can cleave the SP off. A possible mechanism that could prevent the SP cleavage is that the OST complex competes with the SPase. Later when the asparagines are already modified, the bulky sugars could hinder access to the SP cleavage site, leaving the SP of Gp3 uncleaved.

In general it is assumed that SP cleavage occurs before the glycosylation event, early during the translocation, immediately when the SPase gets access to the cleavage site in SP hairpin structure model (Fig. 5.3A) (Steer and Hanover, 1991). However, for some proteins delayed SP cleavage has been observed. In gp160 of HIV the SP is cleaved around 2–3 hours after protein synthesis, when the disulphide bonds are already formed and the protein is folded (Land *et al.*, 2003). Another example is the US11 protein of human cytomegalovirus (HCMV), which downregulates expression of histocompatibility complex (MHC) class I molecules by targeting it to destruction by the proteasome. The SP cleavage of US11 is delayed by an unknown mechanism, but the n part of SP as well the C-terminal transmembrane region are important for this delay As in the Gp3 the N-linked glycosylation was shown to occur co-translationally, it is certain that the OST gains access to the growing polypeptide of Gp3, before the SP cleavage by SPase.

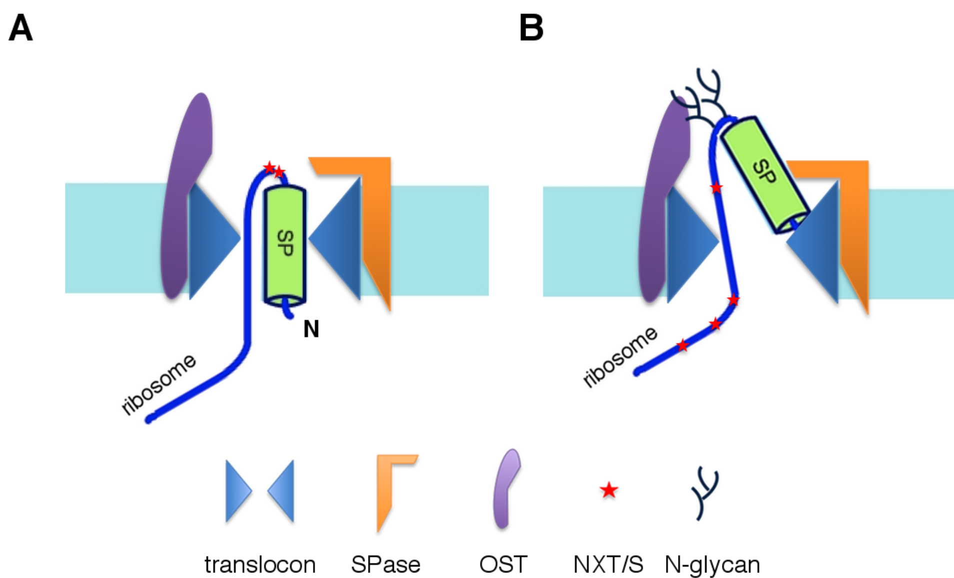

Figure 5.3 Model of interaction of Gp3 with the translocon and associated complexes. (A) The hairpin model of SP association with the translocon channel. Overlapping sequon of the Gp3 is not in the range of OST active site. **(B)** The model in which the SP of Gp3 slips out from the translocon. Overlapping sequon is N-glycosylated and SP cleavage is prevented. N: amino terminus; SPase: signal peptidase; OST: oligosacharyltransferase; NXT/S: N-glycosylation consensus motif; SP: signal peptide.

The OST is associated with the translocon, but the active site of the enzyme is located 15 Å above the membrane, at least for the bacterial homologue of OST (Lizak *et al.*, 2011). Moreover, it has been experimentally shown that in mammalian systems, at least 12 amino acid residues have to be present between the NXT/S sequence and ER membrane (Nilsson and von Heijne, 1993). In Gp3 the NNTT sequon is located two amino acid downstream of the predicted SP cleavage site. This could mean that either OST can glycosylate NNTT sequon of Gp3 although it is too close to the membrane or the SP slips out of the translocon (Fig. 5.3B) The first possibility is unlikely, since sequons located in the proximity of the membrane were shown to be skipped by the translocon-associated STT3A isomer of OST. Such sequons can be modified post-translationally and after SP cleavage by the OST containing another isomer, the STT3B (Ruiz-Canada *et al.*, 2009). As it was discussed in paragraph 5.1, the SP of the Gp3 is not a membrane anchor. Moreover all glycosylation sites in the protein were modified in a co-translational manner (Fig. 2.3A). Therefore, the model that SP slips out of the translocon, allowing access of OST to the N-glycosylation consensus motif, is the most probable one.

5.4 Overlapping NNTT sequon of Gp3 is glycosylated at both asparagines

Another intriguing property of Gp3 is the usage of the overlapping N-glycosylation sequence NNTT. Experimental and bioinformatic approaches showed that sequons that are located in proximity to each other are generally hypoglycosylated (Karamyshev *et al.*, 2005; Shrimal and Gilmore, 2013). In naturally occurring as well as in artificially engineered overlapping sequons, only one was modified. In natural proteins the closest separated sequons, where both asparagines were modified, is the NHSENAT sequence (Karamyshev *et al.*, 2005).

In chapter 2, I demonstrated that in Gp3 the two asparagines in overlapping sequon NNTT are indeed N-glycosylated, although not in every molecule (Fig. 2.1C–D). When the SP of Gp3 was exchanged with the signal anchor and stalk of neuraminidase, only 5 N-linked glycans were present, suggesting that only one of the overlapping sites was glycosylated (Fig.2.1E). It is hard to understand why in Gp3 we observe the usage of both overlapping sequons. The crystal structure of the OST has not been resolved for eukaryotes. The only available data come from the structure of PglB from *Campylobacter*, and AglB from *Archaeoglobus,* the homologues of the catalytic subunit STT3 of OST. The translocated peptide binds to PglB forming a loop that exposes the acceptor asparagine

through a small porthole, pointing it to the oligosaccharide-binding cavity located on the other side of the PglB (Lizak *et al.*, 2011; Matsumoto *et al.*, 2013). The size of the porthole would be the limiting factor in achieving the glycosylation of both asparagines in overlapping sequon in the bacterial homologue of STT3, but it might be different in eukaryotic cells where various compositions of OST complexes were observed (Kelleher and Gilmore, 2006). As the Gp3 with exchanged SP was glycosylated only 5 times, it is likely that surrounding of the acceptor asparagines in the NNTT sequon of Gp3, or behaviour of its SP itself, such as slipping out of the translocon, contribute to simultaneous N-glycosylation of two overlapping sites. In the future it would be interesting to mutate the amino acids sequence in the SP or within the overlapping sequon, e.g. check if the NNSS would also be glycosylated at both asparagines.

5.5 The effect of N-linked glycans adjacent the SP cleavage site of Gp3 on EAV infectivity

In chapter 3 the effect of deletion of the overlapping sequon on the infectivity of EAV was analysed. Similarly to transiently expressed protein, the SP of Gp3 is cleaved in mutant lacking both asparagines on position 28 and 29, but is retained if at least one sugar is added near the SP cleavage site (Fig. 3.2B). This shows that SP processing of the Gp3 observed in transfected cells is also valid for Gp3 expressed during virus infection. Interestingly, the mutations had little effect on virus growth in cell culture (Fig. 3.1B). The content of the major proteins was the same in wt and all mutant viruses, and formation of the minor glycoprotein complex was not altered. The small effect of SP retention in Gp3 on virus replication in cell culture supports the findings from chapter 2 that the SP does not function as a membrane anchor. If the SP anchored the Gp3 to the virion's membrane it would be unlikely that lack of TMD would have no effect on the function of the Gp3. On the other hand, attempts to rescue the virus with hydrophobic C-terminal deletion were unsuccessful, which could indicate that this part is crucial for infectivity of EAV, and support the new topology model.

Because our findings on Gp3 SP retention due to glycans had minimal impact on virus infectivity in cell culture, the question that remains is whether this unique processing of Gp3 could have any advantage for EAV *in vivo*. Among 249 EAV Gp3 sequences from National Center for Biotechnology Information (NCBI), 153 of them possess overlapping glycosylation sequon NNTT, 93 have only one glycosylation site and 3 has no glycosylation

sequons adjacent to the SP cleavage site (see chapter 2). In one study, where EAV was successively isolated from the semen of a persistently infected stallion, the Gp3 sequence possessed the overlapping sequon NNTT. However, subsequent isolation and sequencing of the virus revealed that NNTT had mutated to DNPA, and both of the glycosylation sites were lost, which could indicate that N-glycans on that position are not essential for the maintenance of persistent state in the carrier stallion (Hedges *et al.*, 1999).

The epidemiology and occurrence of new EAV outbreaks is attributed to persistently infected stallions (Miszczak *et al.*, 2012). These carrier animals are the source of new virulent variants of EAV, because despite high neutralizing antibody levels they cannot clear the virus from reproductive system, where the virus replicates and produce quasispecies – viruses with point mutations.

As the ORF3 and ORF4 overlap, one could point out that the evolutionary pressure lies rather in the C-terminus of Gp4, and not in the sequence encoding N-terminal glycosylation sequons of the Gp3. However, the vast majority (98.8 %) of the sequenced EAV strains possesses at least one glycan near the Gp3's SP cleavage site, and therefore retain the SP. Therefore, it is likely that these strains exhibit better fitness in transmission between animals or in establishment of carrier state in the infected stallions. Also other members of *Arteriviridae* family possess one (albeit not two) glycosylation site in proximity of the predicted SP cleavage site (Table 2.1). Although, it is unknown if the same processing of Gp3 applies for other Arteriviruses, this conserved feature in all family members implies that in general it is important.

5.6 Gp2 and Gp4 membrane topology, ER retention and dimerization

In chapter 4, I focused on the two other minor envelope glycoproteins of EAV. Consistent with previously published data and software predictions (de Vries *et al.*, 1995; Wieringa *et al.*, 2002), the membrane topology of Gp2 and Gp4 was confirmed here to be type I. For functional studies, I attempted to express the Gp2/Gp4 dimer on the cell surface. Gp2 and Gp4 with the TMD and cytoplasmic tail of influenza A virus haemagglutinin were able to form heterologous dimers, which passed the quality control in ER and were trafficked to the cis-Golgi, but were not targeted to plasma membrane (Fig. 4.4C). This indicates that the TMD of haemagglutinin is not able to target this complex to plasma membrane.

All structural membrane proteins of EAV have to get to the site of virus assembly and budding, which is undefined internal membrane. When expressed alone, Gp2 and Gp4 localise in ER, but in my experiments simultaneous expression allow dimerization and movement to cis-Golgi. I worked on the constructs with exchanged TMDs, therefore it is unknown if the unaltered Gp2/4 would also reach the cis-Golgi compartment. It is possible that minor glycoproteins interact with each other in order to achieve the correct localization that would result in their incorporation into the budding virus. The E protein was shown to influence the incorporation of the other minor proteins into EAV virus particles (Wieringa *et al.*, 2004). Therefore it is possible that in EAV infection only minor protein complexes that associate with E are trapped on the budding site and incorporated into virions.

As the constructs with modified TMDs could not have been used for the studies on retention of Gp2/4 complex, they were employed to determine which cysteine in Gp4 is responsible for covalent linkage to Gp2. At the same time, the impact of cysteine mutation in Gp4 was investigated in the virus context. The mutation of the cysteine at positions 26 and 77 were lethal for the virus, while the mutation of the cysteine 50 produced a virus with severely affected growth proprieties (Fig. 4.6B), but same mutations in transiently expressed Gp2/4, did not impaired dimer formation (Fig. 4.5).

In contrast to previously described model that each minor glycoprotein in the trimer is linked with one disulphide linkage (Wieringa *et al.*, 2003a) our data suggest that Gp2 and Gp4 might be linked with two covalent bonds, at least in the absence of Gp3 and E. While further experiments need to be conducted to prove this observation, the data described in chapter 4 suggest that more complex interactions between minor glycoproteins may exist.

5.7 Final remarks and outlook

In this thesis I postulated a new topology model of Gp3, which essentially differs from one accepted among other Arteri-virologists. The key feature in this model is that the SP, although uncleaved does not act as a membrane anchor and that the C-terminal HR does not span the membrane completely. It is important to test whether the topology model of Gp3 is also valid for the PRRSV, because the pigs are producing neutralizing antibodies against it. The correct topology might be useful in designing the vector-based or subunit vaccine.

The Gp3 would also be a good candidate for research on translocon–SP interaction.

It would be interesting to investigate which features of SP contribute to full translocation of SP into the ER lumen, and if such mechanism can be seen in other proteins. Moreover, the basis of efficient usage of the overlapping sequon NNTT in the Gp3 needs further investigation and may contribute to the research on OST complex.

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Zusammenfassung

“Membrantopologie und Prozessierung der Glykoproteine Gp2/3/4 des Equinen Arteritis Virus“

Das Equine Arteritisvirus (EAV) ist ein umhülltes RNA Virus aus der Familie der *Arteriviridae,* welches die equine virale Arteritis hervorruft, die mit Fehlgeburten in Stuten einhergeht. Der trimere Komplex, gebildet aus den Glykoproteinen Gp2, Gp3 und Gp4 ist für den Eintritt des Virus in die Zelle verantwortlich. Von diesen Glykoproteinen nimmt man an, dass sie Typ I-Membranproteine sind, die über ein spaltbares Signalpeptid (SP) verfügen.

Um die Orientierung der Proteine in der Membran zu bestimmen, wurde die Lokalisierung eines YFPs, welches C-terminal mit den Glykoproteine fusioniert wurde, untersucht. Im Gegensatz zu Gp2 und Gp4, befindet sich der Fluorophor von Gp3 im Lumen des ERs, was darauf schließen lässt, dass das Signalpeptid als nicht gespaltener Signalanker fungiert (Typ II-Topologie). Die Untersuchung des Glykosylierungsstatus von Gp3 ergab, dass die überlappende Glykosylierungssequenz NNTT, welche sich neben der Spaltstelle des SPs befindet, an beiden Asparaginen modifiziert wird; jedoch nicht in jedem Gp3 Molekül. Das Vorkommen von mindestens einer Glykosylierung in dieser Region ist in der Lage die Signalpeptidspaltung zu verhindern. Die Deletion sowie das Blockieren der Glykosylierung ermöglichen jedoch die Signalpeptidspaltung, was darauf schließen lässt, dass das Anhängen der Zuckerketten die Prozessierung des potentiell spaltbaren SPs verhindert. Dieses Phänomen konnte auch in rekombinanten Viren gezeigt werden, jedoch hatte die Abspaltung des Signalpeptides überraschenderweise keinen Einfluss auf die Infektivität des Virus. Die Untersuchung der Membranbindung in transfizierten Zellen ergab, dass das ungespaltene SP nicht für die Membranbindung verantwortlich ist, sondern der hydrophobe C-Terminus, da seine Deletion die Sekretion des Proteins in den Überstand ermöglichte. Als Ergebnis meiner Untersuchungen stelle ich ein neues Modell für die Topologie von Gp3 auf, in dem das ungeschnittene Signalpeptid vollständig in das Lumen des ERs transloziert wird. Die Membranverankerung findet dabei durch die hydrophobe C-terminale Domäne statt, welche jedoch die Membran nicht komplett durchspannt.

Zusätzlich wurden Konstrukte erstellt, in denen die Transmembrandomänen sowie der zytoplasmatische Teil von Gp2 und Gp4 durch die entsprechende Region vom

Hämagglutinin der Influenzaviren ersetzt wurde. Diese waren in der Lage Gp2/4 Heterodimere zu bilden, wurde jedoch nicht an die Plasmamembran transportiert. Um das für die kovalente Bindung von Gp4 zu Gp2 verantwortliche Cystein zu identifizieren, wurden einzelne Cysteine in der Ektodomäne des Gp4s mutiert und in Zellen exprimiert. Keine der Mutationen was in der Lage die Dimerisierung zu verhindern, allerdings führten die Mutationen im viralen Kontext zu nicht infektiösen Viruspartikeln.

Die präsentierten Daten über EAV könnten die Erforschung des Eintritts der Arteriviren erleichtern. Zudem könnte die Beibehaltung des Signalpeptids, wie es für Gp3 beobachtet wurde, auch für andere Säugerproteine zutreffen. Jedoch sind weitere Studien nötig, um den ungewöhnlichen Mechanismus der Translokation und der Nutzung der überlappenden Glykosylierungssequenz von Gp3 aufzuklären.

Summary

Equine arteritis virus (EAV) is an enveloped RNA virus from the family *Arteriviridae*, which causes equine viral arteritis, mainly associated with abortions in mares. The trimer composed of the minor envelope glycoproteins Gp2, Gp3 and Gp4 is required for cell entry. These glycoproteins are predicted to be type I membrane protein with cleavable signal peptide (SP).

To determine membrane topology in cells, the localisation of YFP fused C-terminally to the glycoproteins was analysed. In contrast to Gp2b and Gp4, the fluorescent tag of Gp3 localised in the lumen of the ER suggesting that the signal peptide (SP) functions as an uncleaved signal anchor (type II topology). The analysis of the glycosylation of Gp3 revealed that an overlapping sequon NNTT, adjacent to the SP cleavage site, was modified at both asparagines, although not in every Gp3 molecule. The presence of at least one glycosylation site in this region prevented SP cleavage in Gp3. Deletion of the overlapping sequon and blocking the glycosylation allowed SP cleavage, indicating that carbohydrate attachment inhibits processing of a potentially cleavable SP. The same phenomenon was observed in recombinant viruses, but surprisingly the infectivity of the EAV lacking the SP was not impaired. Membrane fractionation of transfected cells revealed that the uncleaved SP of the Gp3 was not responsible for membrane attachment of the protein. The membrane anchoring was achieved by the hydrophobic C-terminus, as its deletion allowed secretion of the protein to the supernatant. As a result of my study I propose a new topology model of Gp3, where the uncleaved SP is completely translocated into the ER lumen. The protein is anchored in the membrane via its hydrophobic C-terminus, which however does not span the membrane completely.

Additionally, in order to express the Gp2/4 heterodimer on the plasma membrane for functional studies on this complex, the transmembrane regions and cytoplasmic tails were exchanged with corresponding regions of haemagglutinin of Influenza A. The proteins were able to form disulphide-linked Gp2/4 heterodimers, but were not targeted to the cell surface. In addition, single cysteines in the ectodomain of Gp4 were mutated, to determine the responsible one for the covalent linkage to Gp2. These mutations in transiently expressed Gp2/4, did not abolish dimer formation, but led to non-infectious virions in the viral context.

The presented data about the minor glycoproteins of the EAV may facilitate studies on the Arterivirus entry. The SP retention mechanism observed in Gp3 could also apply for the

other mammalian proteins. Future studies are needed to reveal the molecular basis of the unusual translocation and double sequon glycosylation of the Gp3.

Publications

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 12.12.2013 **Anna Karolina Matczuk**