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Proteolytic processing of the porcine reproductive and respiratory syndrome virus replicase

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ABSTRACT

The porcine reproductive and respiratory syndrome virus (PRRSV) replicase polyproteins pp1a and pp1ab are proteolytically processed by four proteases encoded in ORF1a. In this study, a large set of PRRSV replicase cleavage products were identified and pp1a cleavage sites were verified by using a combination of bioinformatics, proteomics, immunoprecipitation, and site-directed mutagenesis. For genotype 1 PRRSV (isolate SD01-08), proteomic analysis identified H_{180}/S_{181}, G_{385}/A_{386}, and G_{1446}/A_{1447} as the cleavage sites separating nsp1α/1β, nsp1β/nsp2, and nsp2/nsp3, respectively. Transient expression of nsp2-8, nsp3-8, nsp4-8, nsp5-8 (using the recombinant vaccinia virus/T7 RNA polymerase system) and immunoprecipitation identified the cleavage end products nsp2, nsp3, nsp4, nsp7α and nsp7β, and various processing intermediates. Our studies also revealed the existence of alternative proteolytic processing pathways for the processing of the nsp3-8 region, depending on the presence or absence of nsp2 as a co-factor. The identity of most cleavage products was further corroborated by site-directed mutagenesis of individual cleavage sites in constructs expressing nsp3-8 or nsp4-8. This study constitutes the first in-depth experimental analysis of PRRSV replicase processing and the data are discussed against the background of the processing scheme previously derived for the arterivirus prototype, the distantly related equine arteritis virus (EAV). Despite several differences between the two viruses, of which the functional significance remains to be studied, our study demonstrates the general conservation of the replicase pp1a processing scheme between EAV and PRRSV, and likely also the other members of the arterivirus family.

1. Introduction

For over two decades, porcine reproductive and respiratory syndrome (PRRS), a disease first described in the US in 1987 (Keffaber, 1989) and in Europe in 1990 (Wensvoort et al., 1991), has caused tremendous economic losses to the swine industry worldwide, amounting to about $664 million annually in the US alone (Holtkamp et al., 2013). Hallmark symptoms of PRRS are mild to severe respiratory disease in infected newborns and growing pigs, and reproductive failure in pregnant sows. The etiologic agent of the disease, the PRRS virus (PRRSV), is an enveloped, positive-stranded RNA virus that belongs to the order *Nidovirales*, family *Arteriviridae*, which also includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), simian hemorrhagic fever virus (SHFV), and a variety of newly identified simian arteriviruses (Snijder et al., 2013). PRRSV can be divided into distinct European (type 1) and North American (type 2) genotypes that share only about 65% nucleotide identity (Nelsen et al., 1999).

The PRRSV genome is about 15 kb in length and contains at least eleven open reading frames (ORFs; Fig. 1). Of these, ORF1a and ORF1b occupy the 5′-proximal three quarters of the genome and constitute the replicase gene. Together, ORF1a and ORF1b encode two long nonstructural precursor polyproteins, pp1a and pp1ab, with expression of the latter depending on a −1 ribosomal frameshift signal in the short ORF1a/1b overlap region. Following their synthesis from the genomic mRNA template, the pp1a and pp1ab replicase polyproteins are processed into more than a dozen nonstructural proteins (nsps), which assemble into

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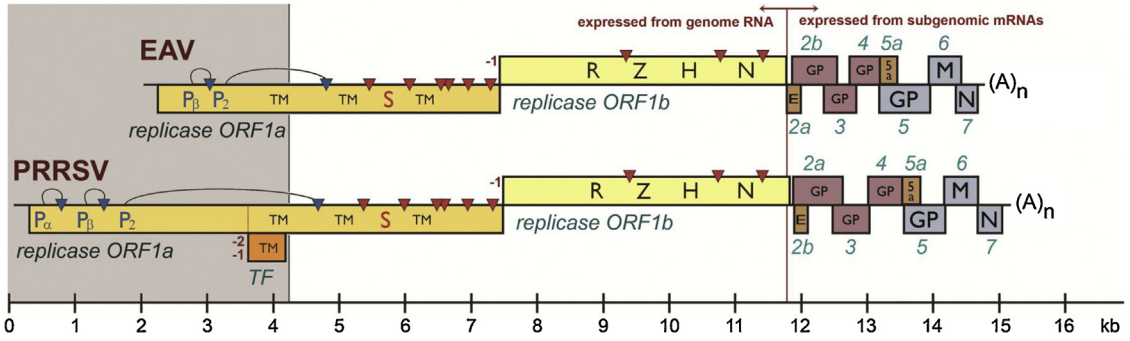

Fig. 1. *Arterivirus genome organization and (predicted) replicase processing scheme.* The genome of the family prototype EAV is shown at the top. The replicase ORFs 1a and 1b (the latter expressed by a −1 ribosomal frameshift) are followed by the genes encoding the minor and major envelope proteins and the N protein. GP, glycoprotein; M, membrane; E, envelope; N, nucleocapsid. Unlike the EAV genome, the genomes of PRRSV and other arteriviruses contain an alternative transframe (TF) ORF underlying the non-structural protein 2 (nsp2)-coding region, which is expressed by −2 ribosomal frameshifting (yielding nsp2TF; Fang et al., 2012), whereas a −1 ribosomal frameshift at the same position yields a third, truncated nsp2 variant (nsp2N; Li et al., 2014). The positions corresponding to (known or predicted) polyprotein cleavage sites are depicted above the replicase ORFs; red arrowheads, sites cleaved by the nsp4 serine protease (S); blue arrowheads, sites cleaved by papain-like proteases (P) in the nsp1-nsp2 region. Three ORF1a-encoded (putative) transmembrane domains (TM) and four highly conserved ORF1b-encoded domains are depicted: RNA-dependent RNA polymerase (R), (putative) multinuclear zinc-binding domain (Z), RNA helicase (H), and NendoU endoribonuclease domain (N).
Modified from Snijder et al. (2013).

a membrane-associated enzyme complex that drives viral RNA synthesis (Fang and Snijder, 2010). PRRSV replicase processing presumably involves a complex proteolytic cascade that is similar to the one previously elucidated for the arterivirus prototype EAV (Snijder et al., 2013; Ziebuhr et al., 2000). However, PRRSV and EAV are only distantly related and both replicase size and sequence have diverged considerably for the two viruses; for example, EAV pp1ab is 3075 residues long, whereas – depending on genotype/strain – PRRSV pp1ab can be between 3800 and 4000 residues. The ORF1b-encoded replicase sequences of the two viruses share ~47% overall amino acid identity (Nelsen et al., 1999) and are predicted to follow the same processing scheme to yield nsp9-12, which include several key functions for viral RNA replication and subgenomic mRNA synthesis (Snijder et al., 2013). The ORF1a-encoded polyproteins, on the other hand, are much more heterogeneous and share only about 28% overall amino acid identity. Important differences in ORF1a expression have already been discovered, including the number of ORF1a-encoded proteases involved, three in EAV in contrast to four in PRRSV. In addition to the papain-like protease (PLP1β) cleaving the nsp1β/nsp2 junction (Snijder et al., 1992), the nsp1 region of PRRSV (and LDV) was found to contain an additional papain-like proteinase (PLP1α), which has apparently lost its activity in EAV and mediates internal cleavage of the PRRSV/LDV nsp1 sequence into nsp1α and nsp1β (den Boon et al., 1995; Sun et al., 2009; Chen et al., 2010;Fig. 1). Recent studies on the SHFV nsp1 region (Vatter et al., 2014) revealed that for this arterivirus, and likely also for several recently identified additional simian arteriviruses (Lauck et al., 2013), the situation is even more complex due to the presence of a third papain-like proteinase and a second additional cleavage site in the nsp1 region. Immediately downstream of the nsp1 region, the N-terminal domain of nsp2 contains another papain-like protease (PLP2; Snijder et al., 1995; van Kasteren et al., 2013), which mediates cleavage of the nsp2/3 junction in addition to playing a role in arterivirus immune evasion by acting as a deubiquitinase and ISG15-deconjugating enzyme (Frias-Staheli et al., 2007; Sun et al., 2010; van Kasteren et al., 2012). The final and "main" arterivirus protease is the chymotrypsin-like serine protease (SP) residing in nsp4 (Snijder et al., 1996; Barrette-Ng et al., 2002; Tian et al., 2009), which is responsible for all proteolytic cleavages downstream of the nsp2/3 junction of both pp1a and pp1ab (Fig. 1; van Aken et al., 2006; van Dinten et al., 1996). In EAV, nsp2 was shown to strongly interact with nsp3 (and nsp3-containing processing intermediates) and to function as a co-factor for cleavage of the nsp4/5 junction by the SP (Wassenaar et al., 1997). This interaction appears to be key to the differential processing of the EAV nsp3-8 region, with the SP either using the 'major' processing pathway (cleavage of the nsp4/5 but not the nsp5/6 and nsp6/7 sites), or the 'minor' processing pathway (nsp4/5 site remains uncleaved, but the nsp5/6 and nsp6/7 junctions are processed).

Recently, an additional important difference segregating EAV and PRRSV (and other arteriviruses) was identified: in all arteriviruses except EAV, the nsp2-coding region of the genome was found to contain a short alternative ORF, termed the transframe (TF) reading frame (Fig. 1), that overlaps ORF1a in the +1/−2 frame. It is expressed by an unprecedented −2 programmed ribosomal frameshifting (PRF) mechanism that generates a transframe protein (nsp2TF) consisting of the N-terminal two thirds of nsp2 fused to an alternative C-terminal domain (Fang et al., 2012). The same PRF signal is also capable of directing −1 PRF, resulting in a third nsp2 variant, a truncated version of nsp2 (nsp2 N), which arises due to the presence of a stop codon in the −1 frame immediately downstream of the PRF signal (Li et al., 2014). Intriguingly, nsp1β was shown to operate as a trans-activator of −2/−1 PRF in the nsp2-coding region (Li et al., 2014). This revealed a unique PRF mechanism, in which a viral protein rather than an RNA structure directs highly efficient non-cannonical translation [PRF efficiencies of ~20 and ~7% have been measured for −2 and −1 PRF, respectively; (Fang et al., 2012)].

Based on the EAV processing scheme and the differences with PRRSV summarized above, maturation of the PRRSV replicase polyproteins is predicted to yield at least 16 products, three more than in the case of EAV, the virus that provided the basis for the currently used numbering and nomenclature of arterivirus nsps (Snijder and Kikkert, 2013; Fang and Snijder, 2010; Ziebuhr et al., 2000). Processing of PRRSV pp1a and pp1ab should yield nsp1 to nsp12, of which (in addition to nsp1) also nsp7 is predicted to be cleaved internally (yielding nsp7α and nsp7β), by analogy with a minor cleavage occurring in the EAV nsp7 region, which was discovered only at a later stage of replicase processing analysis (van Aken et al., 2006). In addition to depending on PRF, the generation of nsp2TF and nsp2N involves the liberation of their N-terminus by the PLP1β-mediated cleavage of the nsp1β/nsp2 site.

In a previous study, using a newly generated panel of PRRSV pp1a-specific monoclonal antibodies, a first analysis of nsp expression in PRRSV-infected cells was performed (Li et al., 2012), but many details of the (predicted) proteolytic cascade used to process the PRRSV replicase remain to be addressed or verified via an