Proteolytic maturation of replicase polyprotein pp1a by the nsp4 main proteinase is essential for equine arteritis virus replication and includes internal cleavage of nsp7

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INTRODUCTION

Equine arteritis virus (EAV), the prototype of the arterivirus family of enveloped, positive-stranded RNA viruses (Snijder & Meulenberg, 2001; Siddell et al., 2005), has a polycistronic genome of about 12.7 kb. Together with the Coronaviridae and Roniviridae, the Arteriviridae are classified in the order Nidovirales (Spaan et al., 2005; Snijder et al., 2005; Gorbalenya et al., 2006). Nidoviruses share a similar genome organization, use common transcriptional and (post)-translational strategies to regulate their gene expression and have a conserved array of homologous replicase domains.

The EAV replicase proteins (nsp1–12) are encoded by two large, 5′-proximal open reading frames, ORF1a and ORF1b, that are translated from the genome RNA, with expression of ORF1b involving a ribosomal frameshift just upstream of the ORF1a termination codon (den Boon et al., 1991). The genome translation products are the polyproteins pp1a (1727 aa) and pp1ab (3175 aa) that are processed by three internal virus-encoded proteinases. nsp1 and nsp2 each contain an autoproteinase domain that liberates these products rapidly from both polyproteins, whereas the nsp4 or ‘main’ proteinase (Snijder et al., 1996; Ziebuhr et al., 2000; Barrette-Ng et al., 2002) is responsible for the processing of all cleavage sites in the remaining parts of pp1a and pp1ab, nsp3–8 and nsp3–12, respectively (Fig. 1) (Wassenaar et al., 1997; Snijder & Meulenberg, 1998; Ziebuhr et al., 2000).

Previously, based on experiments with infected cells and pp1a expression systems, processing of nsp3–8 (and possibly also the nsp3–8 moiety of nsp3–12) was proposed to follow either of two alternative proteolytic pathways (Fig. 1; Wassenaar et al., 1997; Snijder & Meulenberg, 1998; Ziebuhr et al., 2000). In the majority of precursors, the nsp4/5 junction was found to be cleaved, producing the nsp3–4 and nsp5–8 processing intermediates (‘major’ pathway; Wassenaar et al., 1997). Subsequently, the latter product was cleaved at the nsp7/8 site only, whereas the nsp5/6 and nsp6/7 sites were found to remain uncleaved. In the alternative, ‘minor’ pathway, cleavage of the nsp4/5
site was not observed, but instead the nsp5/6 and nsp6/7 junctions in the nsp3–8 and nsp4–8 intermediates were processed. Wassenaar et al. (1997) demonstrated that cleavage of the nsp4/5 site depends on the presence of cleaved nsp2 as a cofactor, but the exact mechanism of this potential regulatory switch in EAV replicase processing remains to be elucidated.

The crystal structure of the nsp4 proteinase (Barrette-Ng et al., 2002) confirmed that its catalytic triad His-1103, Asp-1129 and Ser-1184 is indeed grafted onto a two β-barrel chymotrypsin-like fold (Snijder et al., 1996). The enzyme contains a C-terminal extension (about 45 aa in EAV nsp4), which is found in all nidoviral chymotrypsin-like proteinases, despite their very limited overall sequence similarity (Anand et al., 2002; Yang et al., 2003; Ziebuhr et al., 2003; Smits et al., 2006). Recent studies on this domain of EAV nsp4 revealed that it is not required for proteolytic activity per se, but may be important at a level other than catalysis (van Aken et al., 2006b).

The arterivirus main proteinase displays a preference for Glu at the P1 position and a small amino acid (Gly, Ser or Ala) at the P1′ position [using the nomenclature of Schechter & Berger (1967)] of the substrate (Snijder et al., 1996; Ziebuhr et al., 2000), a specificity typical of picornavirus 3C proteases and related 3C-like enzymes of other RNA viruses (Gorbunova & Snijder, 1996; Ryan & Flint, 1997). As for other positive-stranded RNA viruses (Ding & Schlesinger, 1989; Bartenschlager et al., 1994; Rosé et al., 1995; Hardy et al., 2002), the proteolytic maturation of the replicase is assumed to be crucial for the assembly and function of the enzyme complex that drives arterivirus RNA synthesis.

However, replicase processing in arteriviruses and other nidoviruses has mainly been studied in expression systems and the amount of data from infected cells is very limited. The fact that proteinase inhibitors can block coronavirus replication supported the general importance of pp1a/pp1ab processing for viral RNA synthesis (Kim et al., 1995; Yang et al., 2005), but the importance of individual cleavages has not been addressed in any detail. Reverse-genetics studies demonstrated recently that certain cleavages in the relatively poorly conserved N-terminal domain of the coronavirus replicase pp1a/pp1ab polyproteins may be dispensable for replication (Denison et al., 2004). In contrast, inactivation of two of the three nsp4 cleavage sites (nsp9/10 and nsp11/12) in the most conserved, ORF1b-encoded part of the EAV replicase polyprotein inactivated viral RNA synthesis completely, whereas mutagenesis of the third cleavage site (nsp10/11) allowed RNA synthesis, but blocked the production of infectious progeny (van Dinten et al., 1999).

The importance of the nsp4-specific cleavages in the ORF1a-encoded part of the EAV replicase polyprotein has not been investigated. Mutations blocking each of these cleavage sites were documented previously (Snijder et al., 1996; Wassenaar et al., 1997) but, at that time, the reverse-genetics system required to test their effect and the importance of the two pp1a processing pathways in the context of the viral life cycle was not yet available. We now report that all nsp4-mediated cleavages in EAV pp1a are critical for viral RNA synthesis. In addition, our studies identified a novel cleavage site for the nsp4 proteinase, which is located within the nsp7 subunit and appears to be conserved among arteriviruses. The products of this cleavage had previously gone unnoticed.

Fig. 1. Proteolytic processing of the EAV replicase. (a) Processing map of the 3175 aa EAV replicase polyprotein pp1ab. The three EAV proteinases (PCP, CP and SP), their cleavage sites and the EAV nsp nomenclature are depicted. PCP, nsp1 papain-like cysteine proteinase; CP, nsp2 cysteine proteinase; SP, nsp4 serine proteinase; RdRp, RNA-dependent RNA polymerase; Z, zinc finger; Hel, helicase; N, nidovirus-specific endoribonuclease (NendoU). (b) Overview of the two alternative processing pathways proposed for EAV pp1a (Wassenaar et al., 1997). The association of cleaved nsp2 with nsp3–8 (and probably also nsp3–12) was postulated to be a cofactor in the cleavage of the nsp4/5 site by the nsp4 proteinase (major pathway) (Wassenaar et al., 1997). Alternatively, in the absence of nsp2, the nsp4/5 junction was found to remain uncleaved, but the nsp5/6 and nsp6/7 sites were processed (minor pathway). Adapted from Barrette-Ng et al. (2002).
due to technical reasons. Mutagenesis of the novel cleavage site in the context of the EAV full-length cDNA clone proved to be lethal to the virus.

**METHODS**

**Virus, cells and antisera.** The EAV Bucyrus strain (Doll et al., 1957) was grown in baby hamster kidney cells (BHK-21) as described previously (de Vries et al., 1992). Vaccinia virus recombinant vTF7-3 (Fuerst et al., 1986), which produces the T7 RNA polymerase, was propagated in rabbit kidney (RK-13) cells. The anti-nsp4 and anti-nsp7–8 rabbit sera were originally named anti-5,10 and anti-5,10, respectively (Snijder et al., 1994).

**cDNA constructs.** Mutations were engineered by using standard PCR techniques (Landt et al., 1990). Amplified regions were sequenced fully and standard recombinant DNA techniques were used (Sambrook et al., 1989) to introduce mutations into plasmid pL1a and EAV full-length cDNA clone pEAV1a (see below). Previously described mutations were Ser-1184→Ile, replacing the active-site Ser of the nsp4 proteinase, and five mutations replacing the P1 residue of each of the nsp4 cleavage sites in pp1a (nsp3/4 to nsp7/8) (Fig. 1; Table 1): Glu-1064, Glu-1268, Glu-1430, Glu-1452 and Glu-1677 (Snijder et al., 1998; Wassenaar et al., 1997). Newly generated mutations are listed in Table 1. Most of these were accompanied by a few additional silent mutations to engineer restriction sites that could be used as markers. The wild-type (wt) pL1a expression construct (Snijder et al., 1996) contains the full-length EAV ORF1a (encoding pp1a, comprising nsp1–8) downstream of a T7 promoter and encephalomyocarditis virus internal ribosomal entry site. Mutated PCR fragments were introduced into pL1a by using the unique restriction sites SpeI (nt 3763 in the genome) and EcoRV (nt 4265), and mutations were verified by sequence analysis. pEAV1a is a derivative of EAV full-length cDNA clone pEAV030 (van Dinten et al., 2006b).

**Transient expression and protein analysis.** EAV pp1a was expressed transiently from expression plasmid pL1a in RK-13 cells by using the recombinant vaccinia virus/T7 RNA polymerase expression system (Fuerst et al., 1986) and liposome-mediated transfection (Lipofectamine Plus; Invitrogen) as described previously (van Aken et al., 2006b). Proteins synthesized in transfected cells were labelled with 200 μCi (7-4 MBq) 35S promix ml⁻¹ (Amersham Biosciences) from 5 to 10 h post-vaccinia virus infection. Protocols for cell lysis and immunoprecipitation have been described previously (Snijder et al., 1994). Proteins were separated by SDS-PAGE according to Laemmli (1970) and the amount of 35S label incorporated into protein bands was measured by using a phosphorimager (Bio-Rad Molecular Imager FX) and quantified by using Bio-Rad Quantity One software.

**EAV reverse genetics.** Methods used for transcription of EAV full-length cDNA clones and for transfection of infectious RNA into BHK-21 cells by electroporation have been described previously (van Dinten et al., 1997). RNA quality and yield were assessed by agarose-gel electrophoresis. Virus replication in transfected cells and transfection efficiency were assayed by indirect immunofluorescence assays (IFA) (van der Meer et al., 1998) using a rabbit antiserum directed against replicase subunit nsp3 and a mouse mAb recognizing the nucleocapsid (N) protein. Plaque assays were carried out according to previously described protocols (Snijder et al., 2003).

**Bioinformatics.** Multiple sequence alignments of a representative set of nsp7 sequences from diverse arteriviruses were generated by using the MUSCLE program (Edgar, 2004), taking into consideration the phylogenetic clustering of the Arteriviridae (Snijder et al., 2005; Spaan et al., 2005). The analysis was done using the Viralis platform that integrates software tools and a relational database of virus genome sequences (A. E. Gorbalenya, A. A. Kravchenko, A. M. Leonovich, V. K. Nikolaev, D. V. Samborskiy & V. A. Sorokin, unpublished data).

**RESULTS AND DISCUSSION**

**Both the major and minor pp1a processing pathways are critical for EAV replication**

Previously, EAV replicase pp1a processing was studied by using the recombinant vaccinia virus/T7 RNA polymerase expression system, which was found to reproduce faithfully the cleavages that occur in infected cells. In this expression...

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**Table 1. EAV mutants used in this study and their phenotype**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Wild-type sequence*</th>
<th>Mutated sequence†</th>
<th>Amino acid substitution</th>
<th>IFA nsp3</th>
<th>IFA N</th>
<th>Titre at 13 h p.t.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAV-1a wt</td>
<td>GAAGGGCTA</td>
<td>CCCGGGCTA</td>
<td>Glu-1064→Pro</td>
<td>+</td>
<td>+</td>
<td>1×10⁶</td>
</tr>
<tr>
<td>E1064P</td>
<td>GAAGGGCTA</td>
<td>CCCGGGCTA</td>
<td>Glu-1064→Pro</td>
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<td>−</td>
<td>&lt;2×10⁴</td>
</tr>
<tr>
<td>E1268P</td>
<td>AGAGGAGAGC</td>
<td>AGGCTAGGC</td>
<td>Glu-1268→Pro</td>
<td>−</td>
<td>−</td>
<td>&lt;2×10⁴</td>
</tr>
<tr>
<td>E1430P</td>
<td>GAAGGGAGGA</td>
<td>CCCGGGGGA</td>
<td>Glu-1430→Pro</td>
<td>−</td>
<td>−</td>
<td>&lt;2×10⁴</td>
</tr>
<tr>
<td>E1452P</td>
<td>GAAGATCTTC</td>
<td>CCAAGCTTG</td>
<td>Glu-1452→Pro</td>
<td>−</td>
<td>−</td>
<td>&lt;2×10⁴</td>
</tr>
<tr>
<td>E1677P</td>
<td>GAAGGCTTA</td>
<td>CCCGGGCTA</td>
<td>Glu-1677→Pro</td>
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<td>−</td>
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</tr>
<tr>
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<td>CAGGGCCTG</td>
<td>Glu-1677→Gln</td>
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<td>−</td>
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</tr>
<tr>
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<td>GAGGCCTT</td>
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<td>+</td>
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<tr>
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<td>GAAGCTTT</td>
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<td>GAAGGGCC</td>
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<td>−‡</td>
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<tr>
<td>E1574A/E1575A</td>
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<td>GCAGGAGCC</td>
<td>Glu-1574→Ala/Glu-1575→Ala</td>
<td>−</td>
<td>−</td>
<td>&lt;2×10⁴</td>
</tr>
</tbody>
</table>

*Mutated codons are underlined.
†Mutated codons are underlined; nucleotide changes are indicated in bold.
‡Reversion was observed at 24 h p.t. in one experiment.
system, replacement of the fully conserved P1 Glu residue with Pro was shown to abolish processing of each of the five nsp4 cleavage sites in the nsp3–8 region of pp1a completely (Snijder et al., 1996; Wassenaar et al., 1997). Of these cleavages, the one at the nsp4/5 site was previously designated as a marker for the major processing pathway, whereas processing of the nsp5/6 and nsp6/7 sites was assumed to be unique to the minor pathway (Fig. 1). To assess the significance of individual cleavages in the context of the EAV life cycle and the importance of the major and minor processing pathways in general, we introduced each of these five P1 mutations into an EAV full-length cDNA clone (Table 1). Anticipating a lethal effect of these P1 replacements (van Dinten et al., 1999), the nsp7/8 site was chosen as target to engineer and test some additional, less drastic mutations. P1 residue Glu-1677 was replaced with Gln, which is found at this position at the EAV nsp10/11 junction. The P1’ residue Gly-1678 was replaced with Ala, which is found at this position in various cleavage sites in other arteriviruses (Ziebuhr et al., 2000), and Asn, a mutation that will be discussed in more detail below.

BHK-21 cells were transfected with full-length EAV RNA transcripts specifying these mutations and the phenotype of the mutant viruses was analysed at the end of the first replication cycle (13–15 h post-transfection). Later time points were also monitored to screen for the possible occurrence of reversion in the case of non-viable or crippled mutants. The initial screening was done on the basis of a double IFA using antisera recognizing replicase subunit nsp3 and the N protein, which are reliable indicators of the synthesis of genome and subgenomic mRNA, respectively (Snijder et al., 2003). In addition, transfected cell-culture supernatants were harvested and used for plaque assays to determine infectious-progeny titres.

On the basis of these assays, it was concluded that each of the five P1 Glu→Pro mutations abolished all detectable viral RNA synthesis (Table 1). In repeated experiments, reversion was not observed for any of these mutants (monitored up to 48 h post-transfection). Although we cannot formally exclude additional detrimental effects of these P1 replacements that might affect virus replication, these results and our previous studies (Wassenaar et al., 1997) would suggest strongly that both the major and minor processing pathways (Fig. 1) are critical for EAV RNA synthesis.

Of the additional nsp7/8 cleavage-site mutants, both P1’ mutants (G1678A and G1678N) were viable and produced infectious progeny, although titres were somewhat lower than for the wt control (Table 1). The wt-like phenotype of the G1678N mutant was rather unexpected as, thus far, Asn has not been found at the P1’ position of any (predicted) arterivirus nsp4 cleavage site (Ziebuhr et al., 2000). The non-viable phenotype of mutant E1677Q (Table 1) indicated that Gln is not tolerated at the P1 position of the nsp7/8 site, despite its presence at this position of another EAV cleavage site, the nsp10/11 junction (van Dinten et al., 1999).

Analysis of nsp7/8 mutants in a transient-expression system

To assess their effect on proteolysis of the nsp7/8 junction in some detail, the novel mutations were transferred to pp1a expression vector pL1a. Mutant proteins were expressed transiently, labelled metabolically and processing was analysed by immunoprecipitation with an anti-nsp7–8 serum. This serum specifically brings down the nsp5–8 and nsp5–7 products, of which the latter is thought to be a processing end product (Fig. 1; Wassenaar et al., 1997). Thus, the nsp7/8 cleavage efficiency could be assessed by comparing the amounts of immunoprecipitated nsp5–8 and nsp5–7 (Fig. 2a). As negative controls, the previously described nsp7/8 site P1 mutant (E1677P; Wassenaar et al., 1997) and an nsp4 protease active site mutant (S1184I; Snijder et al., 1996) were included.

The analysis confirmed that – as expected – the P1’ Gly→Ala mutation in viable mutant G1678A did not affect proteolysis of the nsp7/8 junction significantly (Fig. 2, lane 7). However, for the second viable P1’ mutant (G1678N), the Gly→Asn mutation reduced the nsp7/8 cleavage by approximately 80 %. Strikingly, the level of inhibition was comparable to that in non-viable mutant E1677Q (compare Fig. 2a, lanes 5 and 6; the Glu→Gln mutation caused an nsp5–8-specific mobility shift). This suggests that, in the latter mutant, the Glu→Gln replacement in nsp7 may have had additional detrimental effects that could have, for example, affected the functionality of nsp7 or one of its precursors.

Surprisingly, an analysis of the low-molecular-mass region of the gel shown in Fig. 2 revealed additional differences

**Fig. 2.** Immunoprecipitation analysis of the processing of full-length EAV pp1a and several cleavage-site mutants produced in the recombinant vaccinia virus/T7 RNA polymerase expression system (see Methods). (a) nsp7/8 cleavage-site mutants. (b) nsp7/7β cleavage-site mutants. The analysis was performed after a 5 h interval labelling using an nsp7–8 antisera. Mock-transfected cells, wt pL1a and the pL1a-S1184I nsp4 protease mutant were included as controls. The positions of the molecular mass markers used during SDS-PAGE and the various processing products are indicated.
between these mutants. In the lanes of the wt control and all nsp7/8 cleavage-site mutants, the anti-nsp7–8 serum brought down an approximately 14 kDa protein (indicated as p14 in Fig. 2) that was conspicuously absent in the lane of nsp4 active-site mutant S1184I (Fig. 2a, lane 2), suggesting that its presence depended on the activity of the nsp4 proteinase. The generation of this product appeared unaffected by nsp7/8 cleavage-site mutations. Moreover, mutant E1677P (Fig. 2a, lane 4), in which the nsp7/8 cleavage is blocked completely, displayed a second band of approximately 15 kDa (p15). Both products were brought down by an anti-nsp7–8 serum (Fig. 2), but only the ~15 kDa product from mutant E1677P was recognized by an anti-nsp8 serum (data not shown), suggesting that it is a C-terminal pp1a fragment consisting of nsp8 (~5-5 kDa) and a C-terminal fragment of nsp7. This raised the possibility that nsp7 (~25 kDa) was cleaved internally and that p14 in Fig. 2 is in fact the corresponding N-terminal part of the protein. In vitro cleavage assays using EAV nsp4 and substrates from the nsp6–8 region were recently described to yield processing products of similar size (van Aken et al., 2006a).

**Evidence for the generation of smaller products from the nsp7 region in EAV-infected cells**

To investigate whether the novel small products were also produced in EAV-infected cells, the low-molecular-mass region of long exposures of previous immunoprecipitation analyses were scrutinized (Snijder et al., 1994). Indeed, when using the anti-nsp7–8 serum (Fig. 3a, right panel), but not when using the anti-nsp8 serum (Fig. 3a, left panel), at least one and possibly two minor products in the 13–16 kDa size range were detected (indicated by arrows). Previously, these products may have gone unnoticed due to their small size, low abundance and the large amount of the 14 kDa N protein, which is brought down due to its interaction with protein A molecules on the *Staphylococcus aureus* cells used for immunoprecipitation (de Vries et al., 1992). Note that the relatively late appearance of these small products correlated with the accumulation of nsp5–7 and nsp7, suggesting that they may originate from processing of either of these larger intermediates. Also, in Western blot experiments (Fig. 3b), the same small products were recognized by the anti-nsp7–8 serum, but not by the anti-nsp8 serum, again suggesting the generation of previously unrecognized products from the EAV nsp7 region.

**Analysis of internal processing of the nsp7 region in a transient-expression system**

To assess the possibility of an internal cleavage in EAV nsp7 in more detail, we re-evaluated pp1a processing upon transient expression of the wild-type protein and the five P1 Glu→Pro mutants mentioned above (E1064P, E1268P, E1430P, E1452P and E1677P; Table 1) (Snijder et al., 1996; Wassenaar et al., 1997). In particular, we looked for processing products in the 13–16 kDa size range that were recognized by the anti-nsp7–8 serum (Fig. 4a). For convenience, from this point forward, we will refer to the cleavage products representing the N- and C-terminal parts of nsp7 as nsp7α and nsp7β, respectively, and to the

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**Fig. 3.** (a) Pulse–chase experiment analysing expressed proteins of EAV-infected cells immunoprecipitated with an nsp8 antiserum (left panel) and an nsp7–8 antiserum (right panel). (b) Western blot analysis of EAV-infected BHK cells (V) and mock-infected cells (M) with either the anti-nsp8 serum or the anti-nsp7–8 serum 8 h post-infection. Possible minor processing products in the 13–16 kDa size range (indicated by arrows) were detected only by using the anti-nsp7–8 serum.
postulated novel cleavage site as the nsp7α/7β site. Unfortunately, nsp7β (predicted size ~12 kDa) was not detected in our analyses, which may have been due to its low methionine content (Fig. 4b), high turnover or further processing of this product, or to co-migration with background bands present in the gel (Fig. 4a).

As shown in Fig. 2(a), mutant E1677P yielded products of ~14 and ~15 kDa (Fig. 4a, lane 7). The ~15 kDa product was not produced by any of the other constructs, which was in line with the hypothesis that it was the C-terminal nsp7 cleavage product (nsp7β) that could only be visualized when fused to nsp8 (nsp7β–8; Fig. 4b). Also, the incorporation of 35S label into nsp7α and nsp7β–8, as determined by phosphorimager analysis, nicely reflected the methionine content of the two products (3:2; Fig. 4b). The same analysis also revealed an approximately 60% increase in nsp7α level compared with that of the wild-type control, suggesting that the block at the nsp7/8 cleavage site promoted cleavage of the nsp7α/7β junction. For the nsp6/7 cleavage-site mutant E1452P (Fig. 4a, lane 6) the ~14 kDa product shifted to a size of about 16 kDa, suggesting that it indeed represented an N-terminal fragment of nsp7 that had now become N-terminally extended with nsp6 (22 aa; Fig. 4b) to give nsp6–7α. In view of the increased amount of nsp7α in this lane, this mutation also appeared to promote cleavage of the nsp7α/7β site.

The E1452P and E1677P mutants also produced a novel band of approximately 26 kDa (p26) that we cannot explain readily (Fig. 4b). The same product was observed upon expression of several other pp1a mutants with altered processing of the nsp3–8 region (D. van Aken, J. Zevenhoben-Dobbe, A. E. Gorbalenya & E. J. Snijder, unpublished data). The slight migration difference between these two minor bands suggests that the product may in fact contain one of the mutations and thus be derived from the nsp6–8 region itself, e.g. by cleavage at an alternative site when one of the default cleavage sites is blocked. Alternatively, the bands could represent products from another part of the polyprotein or could even be host proteins whose synthesis or co-immunoprecipitation is
Some interesting features of nsp7 in arteriviruses were observed. In the case of E1064P, which does not process the nsp3/4 site, nsp7 accumulated and only a small amount of nsp7 could be observed. In the light of this observation, it is tempting to speculate that nsp7 is derived from a processing step that occurs predominantly in trans and is facilitated by the release of nsp4 from nsp3. This hypothesis is in line with the fact that the cleavage of the nsp3–4 intermediate, which is probably membrane-associated due to the hydrophobic properties of nsp3, is a relatively late event in pp1a maturation (Snijder et al., 1994). Likewise, in mutant E1268P, in which the nsp4 cleavage was blocked and the nsp4 enzyme remained attached to another presumptive transmembrane protein (nsp5), nsp7 was not observed and, also, very little nsp7 had accumulated. Similar processing differences in alternative forms of a viral proteinase have been described previously for the poliovirus 3C enzyme (3C versus 3CD) (Jore et al., 1988; Ypma-Wong et al., 1988) and were, as for our observations, implicated in the fine-tuning of polyprotein processing that is required to regulate the viral life cycle. Formally, we cannot exclude the possibility that the nsp3/4 and nsp4/5 cleavage-site mutations affected the substrate(s) rather than the enzyme necessary to produce nsp7. However, we consider this less likely, as the production of nsp7 was not affected significantly by a block at the nsp5/6 cleavage site (mutant E1430P; Fig. 4a, lane 5), which is closer to nsp7 than the two other cleavage sites probed.

Identification of a putative internal cleavage site in nsp7

The data presented thus far suggested an internal nsp4-driven cleavage in the central region of nsp7. By using the apparent molecular masses of the processing products in Fig. 4(a), we arrived at a processing map for the nsp6/8 precursor that was consistent with all data obtained so far (Fig. 4b). Based on the observed product sizes, the novel internal cleavage site in nsp7 was estimated to be located about 125 aa downstream of the N-terminal Ser-1453 of the protein. Based on the preference of arterivirus main proteinases to cleave between a Glu at P1 and a small amino acid (Gly/Ala/Ser) at P1 (Snijder et al., 1996; Ziebuhr et al., 2000), a potential cleavage site was identified at position E1575/A1576 in EAV pp1a (Fig. 4c). Cleavage of the E1575/A1576 junction in fully processed nsp7 would yield an N-terminal product (nsp7) with a calculated molecular mass of 13.5 kDa, an excellent match with the ~14 kDa product recognized by the anti-nsp7–8 serum in Figs 2–4.

A revised alignment of a representative set of arterivirus nsp7 sequences revealed that this site is located in an extremely divergent region of the replicase polyprotein. Nevertheless, each arterivirus examined was found to have a potential counterpart for this site (Fig. 4c; data not shown). The P1 Glu-1575 matched Glu residues in all other arterivirus sequences, whereas the P1’ Ala-1576 could be aligned with Gly or Asn in the other sequences. Whereas Gly is found at the P1’ position in many arterivirus nsp4 cleavage sites (Ziebuhr et al., 2000), the results presented in Fig. 2 and Table 1 indicated that Asn at P1’ can also be tolerated. Thus, the Glu-1575/Ala-1576 dipeptide was concluded to comply with the P1–P1’ preferences of the arterivirus main proteinase. The poor sequence conservation around this candidate cleavage site explains why it was missed in previous comparative sequence analyses.

To test the above prediction, mutagenesis of the putative nsp7/7β site was conducted, as we anticipated that this would allow us to block off nsp7 production. To this end, a P1 Glu-1575→Ala substitution was introduced into pp1a expression vector pL1a. However, as position 1574 is also occupied by a Glu residue, this change created an alternative potential cleavage site (Glu-1574/Ala-1575) in the mutant (besides the P1–P1’ signature, no other determinants have been identified in arterivirus nsp4 substrates). In order to preclude processing of this alternative substrate, a second Glu→Ala mutation was introduced into an additional construct (double mutant E1574A/E1575A), changing the original Glu–Glu–Ala sequence into Ala–Ala–Ala. The two mutant proteins were expressed transiently as described in Methods and processing of the nsp7/7β site was analysed by immunoprecipitation with the anti-nsp7–8 serum, which had been shown to bring down nsp7 (Fig. 2b).

Indeed, production of nsp7 was affected in both mutants, but was only inhibited completely in the case of the double mutant (Fig. 2b, lane 9). The single Glu-1575→Ala mutation allowed some residual cleavage, possibly – as explained above – due to the cleavage of the artificial Glu-1574/Ala-1575 junction created by the mutation. This would also explain the observation that nsp7 of this mutant, now being 1 aa shorter, migrated slightly faster (Fig. 2b, lane 8). Taken together, our theoretical analysis and experimental data strongly supported the identification of Glu-1575/Ala-1576 as the probable nsp7/7β cleavage site in EAV.

Reverse-genetics data support the importance of the internal processing of nsp7

As described in the first paragraph for the other P1 mutations (at the nsp3/4 to nsp7/8 sites), the effect of mutations at the putative nsp7/7β cleavage site (E1575A and E1574A/E1575A) was tested in the EAV reverse-genetics system (Table 1). In repeated experiments, neither mutant expressed nsp3 or N protein at 13 h post-transfection and did not produce infectious progeny. However, in one experiment, apparent reversion (or pseudoreversion) was observed for mutant E1575A in a 24 h post-transfection experiment, apparent reversion (or pseudoreversion) was observed for mutant E1575A in a 24 h post-transfection IFA, suggesting that this mutant possesses some residual RNA synthesis activity. Thus, the reverse-genetics analysis revealed the general importance of Glu-1575 for EAV viability. On the basis of the data presented in the previous paragraphs, we propose that this is due to its role as P1
residue of the novel nsp7α/7β site, which implies that this relatively late and minor processing step is also critical for virus reproduction.

**Polyprotein pp1a processing is critical for EAV RNA synthesis**

The proteolytic-processing map of the EAV replicase polyproteins was thought previously to be complete. However, several lines of evidence now point towards the occurrence of an additional nsp4-mediated cleavage in nsp7. Although the C-terminal product of this cleavage (nsp7β) was only detected indirectly (when extended with nsp8 in the case of mutant E1677P; Fig. 2a), nsp7α was observed both in infected cells and in expression systems. The position of the novel products in the replicase-processing scheme (Fig. 4b) was supported by their recognition by specific antisera, the size changes upon mutagenesis of flanking cleavage sites (Fig. 4a) and the identification of a conserved potential nsp7α/7β cleavage site (Fig. 4c), whose mutagenesis indeed prevented the production of nsp7α (Fig. 2b). As in the case of the other nsp4 cleavage sites tested in this study (Table 1), mutagenesis of the putative P1 residue (Glu-1575) of the novel site was found to be critical for EAV RNA synthesis.

Mutations in specific EAV replicase subunits were found previously to affect subgenomic RNA synthesis selectively (van Dinten et al., 1997; Tijms et al., 2001). However, our in vivo assessment of the importance of nsp4-mediated processing of the nsp3–8 region showed that mutants in which cleavage of one of the six sites was blocked are all impaired equally in terms of both genome replication and subgenomic mRNA synthesis, suggesting that both the major and the minor processing pathways are essential for viral RNA synthesis. Even more than before, it has become clear that the EAV nsp3–8 region is processed extensively and in a highly regulated fashion to produce a variety of overlapping processing intermediates and relatively small end products.

A wide variety of nsp7-containing cleavage products is produced by arteriviruses

The function of nsp7 in the arterivirus life cycle is currently unknown and so far no homologue of the protein has been identified in other virus systems. The internal processing of nsp7 suggests that nsp7 consists of two smaller subdomains. nsp7α and nsp7β may cooperate to fulfil a joint nsp7 function or may each have a separate function. The number of currently identified processing intermediates that contain EAV nsp7 is remarkable. In certain products (nsp6–8, nsp6–7 and nsp7–8), nsp7 is extended N- and/or C-terminally by the flanking small proteins nsp6 (22 aa) and nsp8 (50 aa), which may thus modulate its function. In the nsp3–8, nsp5–8 and nsp5–7 intermediates, nsp7 is connected to one or multiple hydrophobic domains (nsp3 and/or nsp5; Snijder et al., 1994). Finally, due to its position just upstream of the ribosomal frameshift site, nsp7 is part of large processing intermediates that extend into the ORF1b-encoded part of the replicase, including in particular the nsp9 RNA-dependent RNA polymerase (van Dinten et al., 1996, 1999). Possibly, these long-lived nsp7-containing intermediates play different roles in EAV RNA synthesis.

In the distantly related corona- and toroviruses, the corresponding region of pp1a, which displays no recognizable sequence similarity to that of arteriviruses, is also processed into several small proteins by the main proteinase (Ziebuhr et al., 2000; Gorbalenya et al., 2006; Smits et al., 2006). In coronaviruses, this region encompasses nsp7–nsp10 and it was demonstrated that these proteins colocalize in replication complexes with the viral helicase, N protein and 3C-like proteinase (Bost et al., 2000; Snijder et al., 2006). Recently, genetic studies have implicated nsp10 in viral RNA synthesis (Sawicki et al., 2005) and crystallographic studies showed that nsp9 is an RNA-binding protein (Egloff et al., 2004; Sutton et al., 2004). Furthermore, nsp7 and nsp8 have been shown to form a hexadecameric supercomplex that is capable of encircling RNA and may operate as a cofactor in viral RNA synthesis (Zhai et al., 2005). It will be very interesting to see whether this part of the arterivirus replicase polyprotein, despite the lack of a clear phylogenetic relationship, has evolved to be the functional equivalent of the corresponding region of the coronavirus replicase polyprotein.

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