Mutational analysis of the functional sites in porcine reproductive and respiratory syndrome virus non-structural protein 10

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Porcine reproductive and respiratory syndrome virus (PRRSV) is prevalent throughout the world and has caused major economic losses to the pig industry. Arterivirus non-structural protein 10 (nsp10) is a superfamily 1 helicase participating in multiple processes of virus replication. PRRSV nsp10, however, has not yet been well characterized. In this study, a series of nsp10 mutants were constructed and analysed for functional sites of different enzymic activities. We found that nsp10 could bind both ssDNA and dsDNA, and this binding activity could be inactivated by mutations at Cys25 and His32. These two mutations also abolished unwinding activity without affecting ATPase activity. In addition, substitution of Ala227 by Ser eliminated helicase activity, whilst substitution by Val enhanced unwinding activity. Taken together, our results showed that Cys25 and His32 in PRRSV nsp10 were critical for nucleic acid binding and unwinding, and that Ala227 played an important role in helicase activity.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-sense ssRNA virus, which is classified in the family Arteriviridae, genus Arterivirus along with Equine arteritis virus (EAV), Lactate dehydrogenase-elevating virus (LDV) and Simian hemorrhagic fever virus (SHFV) (Snijder et al., 2013). PRRSV infection is often responsible for severe reproductive failure in sows and gilts, and respiratory problems in pigs of all ages (Music & Gagnon, 2010). In 2006, a highly pathogenic PRRSV variant emerged in China ultimately affecting >2 000 000 pigs with ~ 400 000 fatalities (Tian et al., 2007). The virus genome was found to have a discontinuous deletion of 30 aa in non-structural protein 2 (nsp2), but these deletions may not be associated with the virulence enhancement (Zhou et al., 2009).

ORF1b is the most conserved fragment in the arterivirus genome encoding two key enzymes for virus genome replication: RNA-dependent RNA polymerase (nsp9) and helicase (nsp10) (Fang & Snijder, 2010). The nsp10 helicase of arteriviruses has been reported to participate in multiple viral processes, including subgenomic RNA synthesis (van Dinten et al., 1997), the early steps of mRNA transcription (van Marle et al., 1999), genome replication and virion biogenesis (van Dinten et al., 2000). A comparison between the genomic sequences of attenuated and highly pathogenic PRRSV suggested that mutations in nsp10 may be involved in the virulence of virus (Leng et al., 2012; Li et al., 2010). A recent study also revealed that nsp9 and nsp10 together contributed to the fatal virulence of highly pathogenic PRRSV (Li et al., 2014). A high-resolution structure of the EAV helicase binding to a ssDNA substrate is now available. The nsp10 of EAV appears to be structurally similar to mRNA decay factor Upf1, suggesting that nsp10 plays a role in the post-transcriptional quality control of large viral RNA genomes (Deng et al., 2014).

Arterivirus nsp10 is a superfamily 1 (SF1) helicase comprising a zinc-binding domain (ZBD) and a nucleoside triphosphate-binding/helicase region (Seybert et al., 2000). In previous studies, mutations at 11 of 13 conserved Cys or His residues in the predicted zinc-binding region of the arterivirus nsp10 helicase were found to completely abolish viral RNA synthesis (van Dinten et al., 2000). Some ZBD mutant proteins associated with different EAV phenotypes were found to be ATPase and helicase deficient (Seybert et al., 2005). PRRSV nsp10 has been shown to have ATPase activity and can unwind dsDNA or dsRNA in a 5′→3′ direction (Bautista et al., 2002), but is otherwise uncharacterized. In this study, a series of nsp10 mutants potentially affecting DNA binding, ATP hydrolysis and DNA

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unwinding were constructed, and their biochemical activities were tested to identify critical sites related to different enzymic functions.

The full-length nsp10 gene was amplified by RT-PCR from PRRSV genome RNA (GenBank accession number HM853673) and cloned into the bacterial expression vector pET-30a(+) (Novagen). A soluble protein was expressed in *Escherichia coli* BL21(DE3) pLysS cells (Merck) induced by 0.5 mM IPTG at 16 °C for 16 h. By comparing the conserved amino acids in arterivirus nsp10s (Fig. 1a), 14 mutations (C10A, C25A, H28A, H32A, C41A, H43A, K155A, D225A, E226A, K155A/E226A, A227S, A227V, A228D and A228V) were selected and generated by overlap extension PCR as described previously (Ho *et al.*, 1989). Most mutant nsp10s could be expressed in soluble form, with the exception of C10A, C25A, H28A and H32A (Fig. 1b, lanes 1–12). By fusing to glutathione S-transferase (GST) or small ubiquitin-like modifier (SUMO) proteins, these mutants were also acquired in soluble form at high concentration (Fig. 1b, lanes 14–17). All proteins were purified by Ni²⁺ affinity column chromatography or GST affinity column chromatography, diluted to the same molar concentration, and preserved at −80 °C.

To investigate the nucleic-acid-binding activity of nsp10, Cy5-labelled ssDNA or dsDNA (0.25 μM) was incubated with different concentrations of WT nsp10 (0–20 μM). The Cy5-labelled dsDNA was prepared by mixing a Cy5-labelled ssDNA and its complementary strand (1:1.2), heating to 95 °C, and then slow cooling for annealing. The DNA–protein complex and free DNA were separated by electrophoresis on a 6% non-denaturing gel and traced by fluorography. Distinct bands of the DNA–protein complex could be observed in the gel (Fig. 2a). The intensity of these bands gradually increased with increasing nsp10 concentration. Binding was not observed in the reactions without nsp10 or with BSA (Fig. 2a, lanes 1, 2, 8 and 9). These

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**Fig. 1.** Alignment of four arterivirus nsp10 sequences and SDS-PAGE analysis of the purified nsp10s. (a) The amino acids potentially related to ZBD, ATPase and helicase of nsp10 were compared between EAV (GenBank accession number 23683329), LDV (GenBank accession number 566594645), SHFV (GenBank accession number 25777551) and PRRSV (GenBank accession number HM853673). The mutant amino acids in this study are indicated by grey boxes. Residue numbers in PRRSV nsp10 are labelled along the bottom line and the corresponding amino acids in EAV ORF1ab replicase polyprotein are labelled along the top line. (b) Identification of the purified proteins by SDS-PAGE. Proteins were subjected to 10% SDS-PAGE and stained using Coomassie blue. The molecular mass of the His-nsp10, SUMO-nsp10 and GST-nsp10 proteins was ~49, 68 and 75 kDa, respectively. MW, Molecular mass standards.
results suggested that the PRRSV nsp10 possessed both ssDNA- and dsDNA-binding activities.

To find the crucial amino acids associated with ZBD formation and DNA affinity, nsp10 mutants C10A, C25A, H28A, H32A, C41A and H43A (20 μM) were incubated with 0.25 μM ssDNA or dsDNA. The DNA-binding activity of these proteins was evaluated by electrophoretic mobility shift assay. Proteins altered at C10, H28, C41 or H43 had the same level of ssDNA- (Fig. 2b, lanes 3, 4, 6 and 7) and dsDNA-binding activities (Fig. 2b, lanes 11–14) as the WT protein (Fig. 2b, lanes 1 and 8). However, substitutions at Cys25 and His32 impaired both ssDNA- (Fig. 2b, lanes 2 and 5) and dsDNA-binding activities (Fig. 2b, lanes 9 and 10) as no DNA–protein complex was observed in these cases. The ATPase activity of all these mutant nsp10s was measured by a non-radioactive method as described previously (Fang et al., 2013). The results showed that the ZBD mutants, including C25A and H32A, could hydrolyse as much ATP as the WT control (Fig. 2c), suggesting that these sites had no obvious influence on the ATPase activity of PRRSV nsp10.

To examine the relationship between the ZBD and unwinding activity of nsp10, the helicase activity of ZBD mutant proteins was evaluated. Cy5-labelled dsDNA (0.25 μM) with a 15 nt overhang at the 5′ end was mixed with 6 μM nsp10 in a reaction buffer containing 20 mM HEPES-OH (pH 7.4), 200 mM NaCl, 2.5 mM ATP, 10 % glycerol, 2 mM magnesium chloride, 2 mM DTT and 0.1 mg BSA ml⁻¹ to a final volume of 20 μl, and incubated at 30 °C for 20 min. Reactions were terminated by the addition of 5 μl stop buffer (0.1 M Tris/HCl pH 7.5, 20 mM EDTA, 0.5 % SDS, 0.1 % NP-40 and 0.1 % bromophenol blue). Reaction products were separated on a 10 % non-denaturing polyacrylamide gel and then
Mutant proteins C10A, H28A, C41A and H43A were found to unwind dsDNA (Fig. 3a, lanes 1, 3, 4 and 6) as well as WT nsp10 (Fig. 3a, lane 8). However, the C25A and H32A mutant proteins exhibited an unwinding defect (Fig. 3a, lanes 2 and 5). These results suggested that C25 and H32 were not only crucial for DNA binding, but also necessary for dsDNA unwinding.

Two conserved motifs, GAGKT (aa 152–156) and DEAA (aa 225–228), in PRRSV nsp10 may be involved in ATP binding and hydrolysis. To examine the function of these amino acids, a series of mutants, including K155A, D225A, E226A, K155A-E226A, A227S, A227V, A228V and A228D, were constructed and tested for ATPase and helicase activities. The single mutation at residue K155 was associated with an 88.1 % decrease in ATPase activity, whilst the E226A mutation reduced ATPase activity by 8.0 % (Fig. 3b). All other mutations failed to affect nsp10 ATPase activity (Fig. 3b). Helicase activity of these proteins was analysed by non-denaturing PAGE. As shown in Fig. 3(a), the unwinding ability of K155A (lane 14), D225A (lane 11), E226A (lane 7), A228D (lane 9) and A228V (lane 12) was reduced relative to the WT nsp10 (lanes 8 and 16). Interestingly, the helicase activity disappeared when Ala227 was substituted by Ser (Fig. 3a, lane 10). However, changing the same residue to Val clearly increased helicase activity (Fig. 3a, lane 13). The nsp10 unwinding activity was also quantified by fluorescence resonance energy transfer assay as described previously (Fang et al., 2013). The results were consistent with measurements of helicase activity by non-denaturing electrophoresis (Fig. 3c). Although ATPase activity was defective in the K155A and K155A/E226A mutant proteins, they were able to unwind 45.8 and 58.0 % of the dsDNA substrate, respectively. The D225A and A227S mutations reduced helicase activity by 77 and 90.3 %, respectively, whilst the unwinding ability of the A227V mutant was detected at the same level as the WT protein (Fig. 3c). These results suggested that residues D225 and A227 were important for the unwinding activity of PRRSV nsp10.

Two previous studies of EAV nsp10 examined mutations in ZBD causing different EAV phenotypes and variations in biochemical activity (Seybert et al., 2005; van Dinten et al., 2000). The authors reported that mutant proteins C2377H,
C2395A, H2399C and H2399A were defective in unwinding activity, whilst only H2414C maintained significant ATP hydrolysis and unwinding activities. Based on these results, we selected the corresponding sites in PRRSV nsp10 for mutational analysis and constructed a series of ZBD mutant proteins, including C10A, C25A, H28A, H32A, C41A and H43A. We found that only mutations at Cys25 and His32 impaired the binding and unwinding abilities, whilst the others had no obvious influence on the biochemical activities of PRRSV nsp10. The EAV nsp10 crystal structure shows that 12 Cys or His residues participate in ZBD formation and bind to three zinc ions (Deng et al., 2014). Our results demonstrated that some of these sites are not required for the function of ZBD, but Cys25 and His32 may be critical for forming or maintaining the conformation of ZBD of PRRSV nsp10.

A previous study reported that Zn$^{2+}$ was essential for the refolding of denatured nidovirus helicases, and that ATPase and helicase activities could not be detected if the protein was refolded in a Zn$^{2+}$-free buffer (Seybert et al., 2005). In the present study, the solubility of nsp10 expressed in E. coli was also changed after substituting residues Cys10, Cys25, His28 and His32 with alanine. The results suggested that these amino acids may also influence the folding of nsp10. To obtain a soluble protein in E. coli, we fused these mutant proteins to other tags, such as SUMO and GST. To guarantee that all the results were generated under the same condition, all proteins were used in the same molar concentration in the enzymic assays.

Helicase is known to unwind dsDNA or dsRNA using energy from ATP hydrolysis (Henn et al., 2012; Jankowsky, 2011). Recently, some studies have found that ATP-independent duplex unwinding can be mediated by DEAD-box proteins, but with lower efficiency than ATP-dependent unwinding (Cao et al., 2011; Henn et al., 2010). The binding of ATP to RNA helicase might change the protein’s conformation and enhance RNA–protein binding (Henn et al., 2008). Thus, ATP utilization may not be absolutely necessary for duplex unwinding in some RNA helicases (Henn et al., 2012). In addition, two compounds have been identified to have inhibitory effects on ATPase activity of severe acute respiratory syndrome coronavirus nsp13, which is a virus-encoded SF1 helicase. However, neither compound inhibited unwinding activity (Yu et al., 2012) or has been reported to have an antiviral effect. In the present study, ATPase activity was inactivated in K155A and K155A/E226A mutants, but the two proteins still maintained obvious unwinding activity, although below WT levels. The relationship between ATPase and helicase in PRRSV nsp10 will need to be explored in depth.

Motif II in SF1 helicase is characterized by the highly conserved DExx sequence (Gilhooly et al., 2013; Jankowsky, 2011), in which the x represents any amino acid. Residue Ala227 of nsp10 has low conservation in these helicases. It most probably corresponds to Tyr in PcrA, Ser in Upf1 and Val in RecD2 (Gilhooly et al., 2013). In arterivirus, the amino acid at this site is strictly limited to Ala (PRRSV, LDV and SHFV) or Val (EAV). In our experiments, substitutions at Ala227 had no effect on the ATPase activity of nsp10, suggesting that these proteins folded properly. The unwinding activity of nsp10 disappeared when we substituted Ala227 with Ser, whilst helicase activity was enhanced by substituting Ala227 with Val. These results suggest that residue 227 of arterivirus nsp10 plays a key role in the unwinding process.

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