Virology 411 (2011) 41–49

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Identification of an NTPase motif in classical swine fever virus NS4B protein

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ARTICLE INFO

Article history:
Received 8 October 2010
Returned to author for revision
15 November 2010
Accepted 16 December 2010
Available online 13 January 2011

Keywords:
Classical swine fever virus
NS4B protein
Nucleotide binding domain
NTPase activity
Replication
Virulence

ABSTRACT

Classical swine fever (CSF) is a highly contagious and often fatal disease of swine caused by CSF virus (CSFV), a positive-sense single-stranded RNA virus within the *Pestivirus* genus of the *Flaviviridae* family. Here, we have identified conserved sequence elements observed in nucleotide-binding motifs (NBM) that hydrolyze NTPs within the CSFV non-structural (NS) protein NS4B. Expressed NS4B protein hydrolyzes both ATP and GTP. Substitutions of critical residues within the identified NS4B NBM Walker A and B motifs significantly impair the ATPase and GTPase activities of expressed proteins. Similar mutations introduced into the genetic backbone of a full-length cDNA copy of CSFV strain Brescia rendered no infectious viruses or viruses with impaired replication capabilities, suggesting that this NTPase activity is critical for the CSFV cycle. Recovered mutant viruses retained a virulent phenotype, as parental strain Brescia, in infected swine. These results have important implications for developing novel antiviral strategies against CSFV infection.

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Introduction

CSF is a highly contagious and often fatal disease that affects swine throughout various regions of the world. The etiological agent, CSFV, is an enveloped virus that belongs to the genus *Pestivirus* within the family *Flaviviridae* (Fauquet et al., 2005). The CSFV genome is a positive-sense single-stranded RNA that encodes a single polyprotein precursor that is co- and post-translationally processed by cellular and virus-encoded proteases to produce four structural (C, E^{rns}, E1, and E2) and 8 non-structural (NS) proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Rumenapf et al., 1993; Lindenbach et al., 2007; Thiel et al., 1996). Replication of Pestivirus viral genome is suggested to occur in close association with cytoplasmic membranes through the synthesis of a negative-stranded full-length genome (Gong et al., 1996; Lindenbach et al., 2007). This function is mediated by the activity of NS proteins. NS5B is a viral RNA-dependant RNA polymerase (Steffens et al., 1999; Xiao et al., 2002, 2006); its activity *in vitro* is enhanced by the presence of NS3 protein (Wang et al., 2010). NS3 has multiple functions and is essential for virus replication (Xu et al., 1997). The serine proteinase activity of NS3 is responsible for cleavage of NS4A, 4B, 5A, and 5B and requires the 64 amino acid NS4A protein as a cofactor (Xu et al., 1997; Tautz et al., 1997; Moulin et al., 2007). Additionally, NS3 possesses both nucleoside triphosphatase (NTPase) and RNA helicase activities (Suzich et al., 1993; Tamura et al., 1993; Wen et al., 2007, 2009). The RNA helicase-active portion of NS3 contains a nucleotide-binding motif (NBM) characteristic of all helicases and numerous NTPases (Walker et al., 1982; Gorbalenya and Koonin, 1989). The role of NS2 and NS5A in CSFV replication is less understood. *In vitro* studies using CSFV RNA replicons showed that NS2 is not essential for replication although its presence increased the persistence of RNA replicons in transfected cells (Moser et al., 1999). The role of NS5A in replication may involve at least two different activities: the stimulation of virus replication via formation of multisubunit replication complexes and the inhibition of virus translation (Xiao et al., 2009; Sheng et al., 2010).

The role of NS4B in CSFV replication remains unclear. In Hepatitis C Virus (HCV) and other related flaviviruses, NS4B was found to be an integral hydrophobic membrane protein required for the assembly of "membranous webs" that are derived from endoplasmic reticulum (ER) or the Golgi apparatus and are important for RNA replication (Hugle et al., 2001; Egger et al., 2002; Kim et al., 2004; Miller et al., 2006; Weiskircher et al., 2009). Einav et al. (2004) have shown that HCV NS4B contains a NBM which is conserved among HCV genotypes. This structural motif binds and hydrolyzes ATP, GTP and GDP and also possesses adenylate kinase activity (Einav et al., 2004; Thompson et al., 2009). Amino acid substitutions in the NBM of HCV NS4B impaired NTP binding and hydrolysis and resulted in decreased NTPase and adenylate

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0042-6822/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2010.12.028

kinase activity (Einav et al., 2004; Thompson et al., 2009). Furthermore, the NBM was shown to be critical for the role of NS4B during *in vitro* HCV replication (Einav et al., 2004; Lindstrom et al., 2006) and in cellular transformation and tumor formation (Einav et al., 2008).

Conserved sequence elements in NBM-containing proteins that possess NTPase activity harbor characteristic Walker A and Walker B motifs. The Walker A motif consists of a G-rich phosphate-binding loop with a consensus sequence G/AXXXXGKS/T (where X could be any residue) that is involved in binding of β- and γ-phosphates of NTPs. The Walker B motif consists of an Asp residue preceded by a stretch of hydrophobic amino acids (h) hhhhD or hhhhDD/E, that chelates the Mg^{2+} of the Mg–NTP complex (Walker et al., 1982; Gorbalenya and Koonin, 1989; Mimura et al., 1991; la Cour et al., 1985; Pai et al., 1989). In this study, data is presented demonstrating that CSFV NS4B has NTPase activity. *E. coli* expressed His-tagged CSFV NS4B purified protein hydrolyzes both ATP and GTP. Conserved Walker A and B motifs, characteristic of NBMs in CSFV NS4B (residues 209–216 and 335–342, respectively), are shown to be highly conserved in NS4B proteins of the other pestiviruses, Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV). It is also demonstrated that the enzymatic activity of the protein is affected by specific residue substitutions within identified Walker A and B motifs. Additionally, we have observed that NS4B of pestiviruses lacks the universally conserved K residue in the GKS/T signature of A motifs, but a highly conserved K_{206} residue is located at the N-terminal end of the canonical motif that has an effect on the NTPase activity of the protein. Additional results suggest that specific substitutions within the canonical Walker A and/or Walker B motifs in NS4B are deleterious for CSFV, while a CSFV Walker A revertant virus or viruses harboring mutations at K206 were viable and retained a virulent phenotype in infected swine.

Results

Identification of a nucleotide-binding motif in NS4B

Conserved sequence elements in NBM-containing viral proteins include both Walker A and Walker B motifs (Walker et al., 1982; Gorbalenya and Koonin, 1989) (Fig. 1). Amino acid sequence analysis of NS4B proteins showed that pestiviruses possess sequences resembling canonical Walker A (G/AXXXXGKS/T) and Walker B (hhhhD or hhhhDD/E) motifs (Figs. 2 and 3A). These sequences are located towards the C-terminal portion of CSFV NS4B, encompassing amino acid residues 209–216 and 335–342 of the protein, for A and B motifs respectively. Furthermore it was observed that the putative Walker A motif in pestivirus NS4B lacks the universally conserved K residue in the GKS/T signature sequence, where K has been substituted to V or I (Figs. 2 and 3A). Instead, NS4B of these viruses has a conserved K residue (K206 in CSFV) at the N-terminal end of the Walker A motif, resembling the position of the K residue in deviant Walker A motifs found in ATPase catalytic centers of phage terminases (Mitchell and Rao, 2004) and some prokaryotic and yeast DNA-dependent ATPases or GTPases (Koonin, 1993a, 1993b) (Fig. 3B). The putative Walker B motif in CSFV NS4B, $LLGVDSEG_{335-342}$, concurs with the consensus hhhhD (Fig. 3A). The motif is also conserved among other pestiviruses (Fig. 3A). The sequence $DSEG_{339-342}$ within the B motif matches the consensus DXXG found in highly conserved GTP-binding protein families including elongation factors, ras p21, phosphoenolpyruvate carboxykinase and guanine nucleotide-binding proteins of adenylate cyclase (G proteins) (Dever et al., 1987) (data not shown).

CSFV NS4B has NTPase activity

His-tagged CSFV strain Brescia NS4B protein was expressed in *E. coli* (Fig. 4A), and purified to near homogeneity using cobalt immobilized affinity chromatography (IMAC) resins. Protein purity was verified by Coomassie blue staining (Fig. 4B) and Western blot using an anti-poly-His monoclonal antibody (data not shown). Assessment of the ATPase and GTPase activities of wild-type purified CSFV NS4B was performed by evaluating the release of inorganic phosphate using a colorimetric assay as described in Materials and Methods. Consistent with the sequence analysis, purified wild-type NS4B catalyzed the hydrolysis of ATP and GTP (Fig. 4C). Time course analysis of ATP and GTP hydrolysis by wild-type NS4B revealed that the enzymatic activity achieved maximum kinetics after 2 h of incubation with ATP or GTP (Fig. 4C). Data suggest a

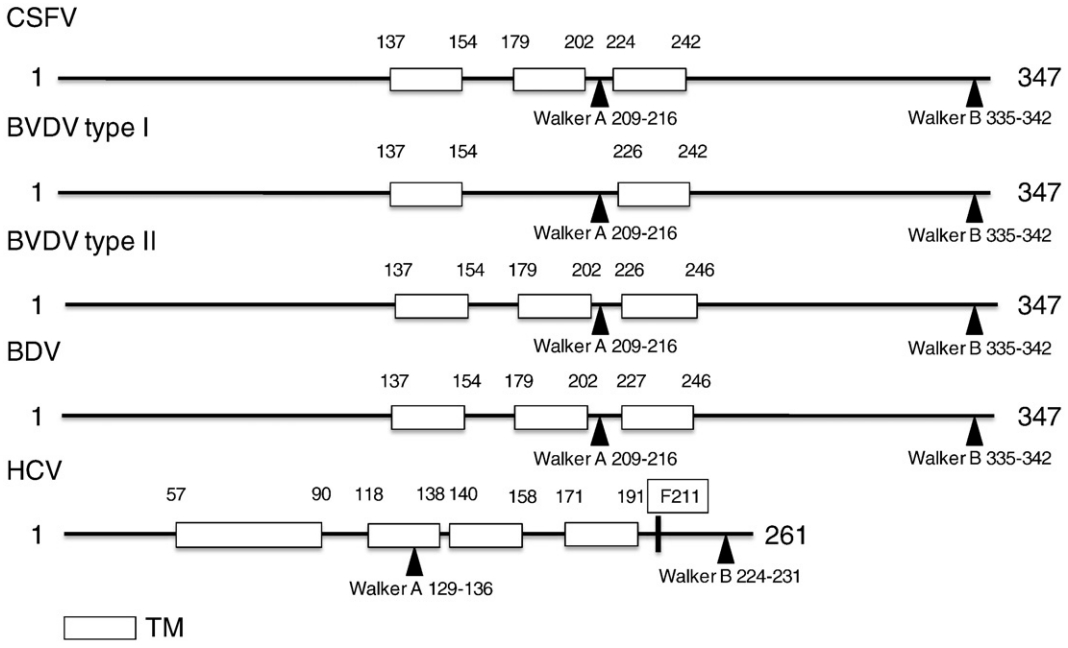

Fig. 1. Schematic representation of NS4B protein from CSFV, BVDV types I and II, BDV, and HCV genotype 1b showing predicted membrane-spanning regions (TM) according to Hofmann and Stoffel, 1993 (TMpred program). Arrowheads indicate locations of Walker A and B motifs. For HCV F211 is the nucleotide binding motif G (Thompson et al., 2009). Numbers indicate amino acid residues.