

Chapter 5

HCV NS2/3 Protease

Sarah Welbourn and Arnim Pause

ABSTRACT

The hepatitis C virus NS2/3 protein is a highly hydrophobic protease responsible for the cleavage of the viral polypeptide between non-structural proteins NS2 and NS3. However, many aspects of the NS2/3 protease's role in the viral life cycle and mechanism of action remain unknown or controversial. NS2/3 has been proposed to function as either a cysteine or metalloprotease despite its lack of sequence homology to proteases of known function. In addition, although shown to be required for persistent infection in a chimpanzee, the role of NS2/3 cleavage in the viral life cycle has not yet been fully investigated due to the lack of an *in vitro* system in which to study all aspects of HCV replication. However, several recent studies are beginning to clarify possible roles of the cleaved NS2 protein in modulation of host cell gene expression and apoptosis.

INTRODUCTION

The NS2/3 protease is the first of two virally encoded proteases required for HCV polyprotein processing. Extending from amino acids 810-1206, NS2/3 is the first non-structural (NS) protein translated and is responsible for the intramolecular cleavage between NS2 and NS3 (see Fig. 1). The amino terminus of NS2 is cleaved from the adjacent p7 protein by host signal peptidases in a membrane-dependent

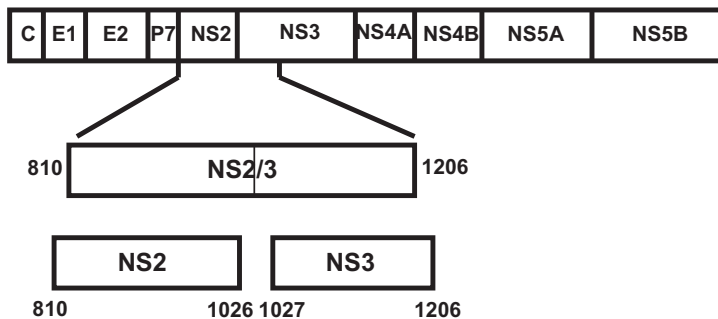


Fig. 1. The HCV NS2/3 protease. The NS2/3 protease is shown in the context of the HCV polyprotein. NS2 and the protease domain of NS3 (from aa 810 to 1206) constitute NS2/3, which undergoes autocatalytic cleavage between aa 1026 and 1027.

manner, while the chymotrypsin-like serine protease located in NS3 is responsible for the cleavage at the NS3/4A and downstream junctions. The HCV NS2/3 protein is an autoprotease whose activity is separate from NS3 protease functions (see Chapter 6). Although many studies have focused on the residues and sequences required for efficient NS2/3 processing, the exact nature of the protease has still not been firmly established, with it being proposed to function as either a novel cysteine or metalloprotease. Furthermore, although all NS proteins are proposed to play a role in viral replication, the exact functions of HCV NS2/3, as well as cleaved NS2 remain largely unexplored; however, some interesting potential functions have emerged in recent years. This chapter will focus on the known properties of the NS2/3 protease as well as the possible functions of both the NS2/3 protease and the NS2 protein.

NS2/3 CATALYTIC CLEAVAGE

GENERAL STRUCTURAL FEATURES OF NS2/3

The NS2/3 protease is responsible for the intramolecular cleavage of NS2 from NS3 between aa 1026 and 1027 (Grakoui et al., 1993; Hijikata et al., 1993a). Fig. 2 shows the main structural and functional domains of the protein. NS2 contains a highly hydrophobic N-terminal region suggested to contain multiple transmembrane segments; however, this region is not required for efficient cleavage at the NS2/3 site (Hijikata et al., 1993a; Pallaoro et al., 2001; Thibeault et al., 2001). The minimal domain for activity of the enzyme has been mapped to aa 907-1206 (Pallaoro et al., 2001). This encompasses the C-terminal portion of NS2, immediately following the hydrophobic region, as well as the N-terminal protease domain of NS3. Although these sequences are required and sufficient for cleavage activity, processing is not dependent the NS3 serine protease activity (Grakoui et al., 1993; Hijikata et al., 1993a). This differs from the NS2B protein of flaviviruses, in which the NS3

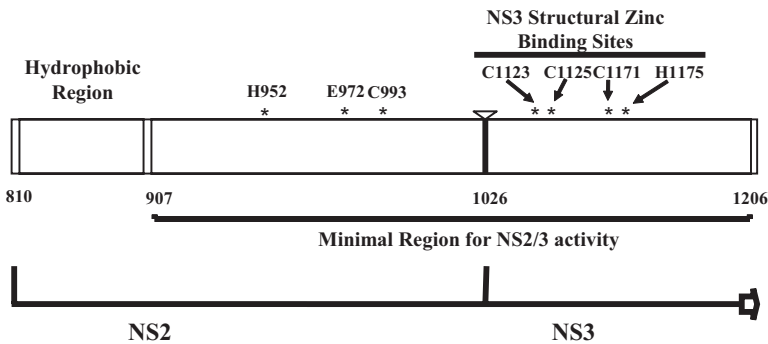


Fig. 2. Functional domains of the NS2/3 protease. The NS2/3 protease encompasses an N-terminal hydrophobic region, with a minimal domain required for activity between aa 907 and 1206. Residues in NS2 required for NS2/3 processing (H942, E972, C993), as well as residues in NS3 responsible for the coordination of a zinc atom are shown.

protease performs a *cis*-cleavage at the NS2B site and then uses NS2B as a co-factor for the processing of the downstream NS polypeptide (Chambers et al., 1991; Chambers et al., 1990; Falgout et al., 1991).

NS2/3 PROCESSING REQUIREMENTS

The HCV NS2/3 protease shows no sequence motifs typical of known proteases; however, sequence alignments show similarity with the GBV NS2/3 protein as well as the bovine viral diarrhea virus (BVDV) NS2/3 protein (Lackner et al., 2004). Residues H952, E972 and C993 are conserved among all genotypes of HCV and mutation of H952 or C993 to alanine completely inhibits NS2/3 cleavage activity while a glutamic acid 972 to glutamine substitution also significantly affects processing (Grakoui et al., 1993; Hijikata et al., 1993a). Furthermore, although NS3 serine protease activity is not required for NS2/3 processing, the full NS3 protease domain must be present and cannot be substituted for another NS protein (Santolini et al., 1995). In addition, mutation of cysteine residues 1123, 1127 and 1171 in NS3, which together with H1175 participate in the coordination of a zinc molecule (Kim et al., 1996; Love et al., 1996), abolishes both NS3 and NS2/3 activities (Hijikata et al., 1993a), presumably by disrupting folding of the enzymes. This therefore suggests that the NS3 protease domain is required to play a structural role in the folding of the enzyme.

Proper folding of the NS2/3 protein and cleavage site plays an important role in the efficiency of NS2/3 processing. Residues surrounding the cleavage site, WRLLD↓APIT, are highly conserved between HCV genotypes but are remarkably resistant to mutations (Hirowatari et al., 1993; Reed et al., 1995). Only mutations severely affecting the conformation of the cleavage site (such as deletion or proline substitution of P1 or P1') severely inhibit cleavage. Furthermore, NS4A-derived peptides that upon binding cause a conformational rearrangement of the NS3 N-terminus are potent inhibitors of NS2/3 activity, likely by altering the positioning of the cleavage site (Darke et al., 1999; Thibeault et al., 2001). The presence of microsomal membranes or non-ionic detergents has been found to be required for *in vitro* processing at the NS2/3 site in certain genotypes (Pieroni et al., 1997; Santolini et al., 1995), while increasing the efficiency of cleavage of others (Grakoui et al., 1993; Santolini et al., 1995), suggesting the hydrophobic environment is necessary for proper folding of the enzyme and positioning of the cleavage site. Similarly, Waxman et al. (2001) have demonstrated the requirement for the ATP hydrolyzing ability of molecular chaperone HSP90 for efficient cleavage in *in vitro* and cell based assays. A similar phenomenon has been described for the BVDV NS2/3 protein where a cellular DnaJ chaperone protein, Jiv, has been found to associate with and modulate NS2/3 activity, possibly by causing a conformational change in the protein (Rinck et al., 2001). Although the mechanisms are still unclear, this could point to a role of cellular chaperones in inducing/maintaining the proper conformation of NS2/3 required for cleavage.

MECHANISM OF ACTION: CYSTEINE OR METALLOPROTEASE?

Initial studies showing NS2/3 activity is inhibited by EDTA and stimulated by zinc led to the early suggestion that NS2/3 functions as a zinc-dependent metalloprotease (Hijikata et al., 1993a). However, with the discovery of the importance of zinc for the structural integrity of the NS3 protease domain, others have proposed NS2/3 may be a novel cysteine protease with a catalytic dyad comprised of H952 and C993 with the possible involvement of E972 as the third residue of a catalytic triad. Inhibition studies both in *in vitro* translation systems and with purified proteins have failed to yield a definite classification (Pallaoro et al., 2001; Pieroni et al., 1997; Thibeault et al., 2001). Although inhibited by metal chelators such as phenanthroline and EDTA, this inhibition is relieved by the addition of ZnCl₂, CdCl₂ or MgCl₂. This could therefore point to a structural rather than catalytic role for the zinc molecule as Cd has not traditionally been able to functionally replace Zn in other metalloproteases (Angleton and Van Wart, 1988; Cha et al., 1996; Holland et al., 1995). However, although classical cysteine protease inhibitors iodoacetamide and N-ethylmaleimide show strong inhibition of NS2/3 processing, no single cysteine has been found to be more susceptible to these alkylating agents (Pallaoro et al., 2001).

Recently, conserved His, Cys and Glu have also been found to be present in BVDV strains and required for NS2/3 cleavage *in vitro* (Lackner et al., 2004), suggesting a similar mechanism of action of the two proteases. However, several differences exist. In addition to the necessity of the N-terminal hydrophobic region of NS2, BVDV NS2/3 does not require the full NS3 protease domain for activity, but rather possesses a conserved zinc-binding site within NS2 itself (Lackner et al., 2004). Although no traditional metal-binding sequences have been identified in HCV NS2, the presence of an additional catalytic zinc in NS2 or a catalytic role for the NS3 zinc molecule cannot be definitely ruled out. The elucidation of the so far unknown crystal structure of NS2/3 should bring important insights into the mechanism of cleavage of this enzyme.

NS2/3 BIMOLECULAR CLEAVAGE

Bimolecular cleavage of NS2/3 has been shown to occur, albeit inefficiently, in cell transfection experiments (Grakoui et al., 1993; Reed et al., 1995). In this system, NS2/3 proteins with mutations/deletions in either the NS2 or NS3 domains could support cleavage provided the missing functional region was co-expressed on a separate polypeptide. In addition, catalytically inactive NS2/3 mutants were also found to inhibit processing of a wild-type protein when expressed in *trans*. The observation that a recombinant NS2/3 protein forms dimers *in vitro* is consistent with these findings (Pallaoro et al., 2001). However, no *trans* cleavage has been observed using purified proteins (Pallaoro et al., 2001; Thibeault et al., 2001). Interestingly, NS2/3 activity *in vitro* was found to be concentration dependent, supporting the notion that dimer formation is essential for the reaction (Pallaoro et al., 2001). Dimitrova et al. (2003) have also demonstrated the homo-association of

the NS2 protein in various systems and suggest that the cleavage between NS2 and NS3 could potentially be performed by dimers of NS2/3 encoded on neighbouring polyprotein chains. As NS2/3 cleavage is widely believed to be an intramolecular event, the significance of bimolecular cleavage in the polyprotein processing events of HCV infection *in vivo* remains to be determined.

ROLE OF NS2/3 CLEAVAGE IN VIRAL REPLICATION

The role of the NS2/3 protease in HCV replication remains to be fully understood. NS2/3 cleavage is required for viral replication *in vivo*, as demonstrated by an HCV clone devoid of NS2/3 activity that fails to cause a persistent infection in a chimpanzee (Kolykhalov et al., 2000). However, NS3-3' UTR subgenomic replicons not encoding the NS2 protein replicate efficiently in Huh-7 cells (Lohmann et al., 1999), suggesting NS2/3 is not strictly required for genome replication.

If cleavage at the NS2/3 site occurs solely for the release of the NS2 protein, what is the advantage for the virus of encoding two distinct proteases for polyprotein processing? Although several roles have been proposed for the cleaved NS2 protein, the NS2/3 protease itself appears unique in that its activity subsequently causes its inactivation. However, potential regulation of the cleavage reaction could have other implications for the viral life cycle, as is known for BVDV NS2/3 processing. BVDV strains are present in two forms, non-cytopathic (noncp) which expresses primarily uncleaved NS2/3 and has the ability to cause persistent infection and cytopathic (cp) strains expressing cleaved NS3 (Donis and Dubovi, 1987; Pocock et al., 1987). For this pestivirus, RNA replication levels have been shown to correlate with amount of cleaved NS3 protein (Lackner et al., 2004), whereas the uncleaved NS2/3 is required for viral infectivity (Agapov et al., 2004). Evolution of a cp strain from a non-cp strain occurs through the activation of the NS2/3 cleavage by a variety of mutations, deletions, duplications and rearrangements within the NS2 region (Kummerer et al., 1998; Meyers et al., 1992; Tautz et al., 1996; Tautz et al., 1994). However, it has recently been suggested that BVDV NS2/3 is an autoprotease whose temporal regulation is involved in modulating the different stages of RNA replication and viral morphogenesis (Lackner et al., 2004). Whether HCV NS2/3 could perform a similar regulatory role remains to be determined. Although NS2/3 processing appears to be a very efficient event in cell expression systems, the possible role for an uncleaved NS2/3 precursor in the complete viral life cycle has not been ruled out.

NS2 AS PART OF THE REPLICATION COMPLEX?

HCV RNA replication has been proposed to occur via the formation of a membrane bound replication complex that comprises the association of the NS proteins required for genome replication (NS3-5B). However, due to the lack of an efficient cell culture system to study the viral life cycle, studies focusing on the

replication complex have been so far limited to the subgenomic replicon system (see Chapter 11), where NS2 is not expressed. Several studies have indicated that NS2 is an integral membrane protein that is targeted to the endoplasmic reticulum (ER) (Santolini et al., 1995; Yamaga and Ou, 2002). Interestingly, NS2 has been found by one group to be inserted into the membrane only when expressed in the context of the NS2/3 protein, and only after cleavage from NS3 (Santolini et al., 1995). NS2 has also been found to interact with all other HCV NS proteins in *in vitro* pull-down, as well as cell-based co-localization and co-immunoprecipitation experiments (Dimitrova et al., 2003; Hijikata et al., 1993b). Therefore, although not required for RNA replication, the possible presence of NS2 in this complex as an accessory protein is plausible and warrants further investigation.

ROLES OF CLEAVED NS2

HCV NS2 IS AN INTEGRAL MEMBRANE PROTEIN

The NS2 protein derived from the cleavage of NS2/3 is inserted into the ER membrane through its N-terminal hydrophobic domain. However, the exact mechanisms of translocation as well as the membrane topology of the protein remain controversial. Membrane association has been found to be dependent on SRP-SRP receptor targeting (Santolini et al., 1995). It was originally proposed that a signal sequence present in upstream p7 was required for membrane association co-translationally, although NS2 translocation has subsequently been demonstrated by several groups to be p7 independent (Santolini et al., 1995; Yamaga and Ou, 2002). Furthermore, although the cleavage at the p7-NS2 junction is performed in a membrane-dependent fashion by signal peptidase (Lin et al., 1994; Mizushima et al., 1994) and the presence of membranes is stimulatory (and for some strains required) for NS2/3 cleavage, one group has shown that the integration of NS2 into the membrane is performed post-translationally, and only after cleavage from NS3 (Santolini et al., 1995). However, Yamaga and Ou (2002) have since proposed that translocation could occur co-translationally and therefore the exact mechanisms of integration remain unclear. The amino terminal region of NS2 is likely to span the membrane several times (Pallaoro et al., 2001; Yamaga and Ou, 2002). However, the exact number of transmembrane domains, as well as the orientation of the protein in the membrane have not been conclusively determined.

NS2 AND NS5A HYPERPHOSPHORYLATION

HCV NS5A has many roles in both RNA replication and the modulation of the host cell environment during infection and has been found to be present in two distinct phosphorylated forms: p56 and p58 (see Chapter 9). Liu et al. have reported the importance of NS2 for the generation of hyperphosphorylated NS5A (p58) (Liu et al., 1999). Using plasmids expressing various sections of the HCV polyprotein in transient transfection experiments, they demonstrate the requirement of NS2 generated by the cleavage of NS2/3 for the formation of p58. However, while

performing similar experiments, other groups have demonstrated the appearance of p58 without the presence of NS2 (Koch and Bartenschlager, 1999; Neddermann et al., 1999). Indeed, Neddermann et al. (1999) therefore suggested that NS2 itself is not required for the hyperphosphorylation process, but rather that it could be the authentic N-terminus of NS3, generated by NS2/3 cleavage, that is of importance.

NS2 INHIBITION OF GENE EXPRESSION

NS2 may also play a role in modulating cellular gene expression in infected cells. One study by Dumoulin et al. (2003) found that NS2 exerted a general inhibitory effect on the expression of a reporter gene expressed from a variety of different promoters (human ferrochelatase promoter, NFkappaB binding sites, SV40 promoter/enhancer sequences, full length, as well as minimal TNF-alpha promoters and cytomegalovirus immediate-early promoter) in several different hepatic and non-hepatic cell types. The amino-terminal (810-940) region of NS2 was sufficient to cause this effect, suggesting inhibition of gene expression is not dependent on the activity of the NS2/3 protease itself. It was therefore suggested that NS2 could potentially regulate host cell protein levels by interfering with a general aspect of transcription or translation. Indeed, several other HCV-encoded proteins, including core (Chapter 3), NS4B (Chapter 8) and NS5A (Chapter 9), have been demonstrated to alter cellular gene expression through a variety of mechanisms (Kato et al., 1997; Kato et al., 2000; Naganuma et al., 2000; Ray et al., 1995). This aspect of NS2 function will require further confirmation and careful investigation as it indicates a potential role for NS2 in the modulation of the host cell environment which has important implications for both the establishment of persistent infection and the pathogenesis of chronic HCV.

NS2 AND APOPTOSIS

In order to establish a persistent infection, many viruses have evolved mechanisms to interfere with cellular apoptosis. In this manner, the virus is then able to replicate to sufficient levels without the elimination of the host cell. Several HCV proteins have been implicated in the modulation of cell signalling and apoptosis, including core, E2, NS5A and NS2 (Gale et al., 1997; Honda et al., 2000; Machida et al., 2001; Ruggieri et al., 1997). Machida et al. (2001) have reported that Fas-mediated apoptosis is inhibited in transgenic mice expressing HCV core, E1, E2 and NS2 proteins. The expression of these proteins in the liver prevented cytochrome c release from the mitochondria as well as preventing the activation of caspase 9 and caspase 3/7, but did not affect caspase 8. Therefore, this implicates these HCV proteins in the mitochondrial intrinsic apoptotic pathway, which involves mitochondrial membrane permeabilization and the release of pro-apoptotic factors, resulting in cell death. Furthermore, Erdtmann et al. (2003) showed that NS2 inhibits CIDE-B-induced apoptosis in co-expression experiments. CIDE-B (cell death-inducing DFF45-like effector) is a mitochondrial pro-apoptotic protein whose overexpression has

been shown to induce cell death (Inohara et al., 1998). CIDE-B-induced apoptosis requires mitochondrial localization and dimerization of the protein, both of which are mediated by a region in its C-terminal domain (Chen et al., 2000). NS2 was found to interact specifically with the C-terminal region of CIDE-B and block cytochrome c release from the mitochondria as well as cell death (Erdtmann et al., 2003). NS2 could therefore potentially prevent the dimerization of CIDE-B required for activity. However, the mechanism of inhibition remains unclear as NS2 is thought to be localized at the ER membrane. In this case, NS2 could potentially bind and sequester CIDE-B, preventing its localization at the mitochondria.

The roles of mature cleaved NS2 remain largely unexplored. Although some possible functions have been proposed and are described here, the lack of an efficient cell culture system remains a major hurdle in identifying the main tasks of NS2 in the various events of the viral life cycle. Furthermore, it has been observed that NS2 is a short-lived protein in replicon cells (Franck et al., 2005). Franck et al. (2005) showed that NS2 is a target for phosphorylation by CK2 and is subsequently rapidly degraded by the proteasome. This appears to be a ubiquitin-independent process and the exact mechanisms involved have yet to be identified. However, the regulation of this process could have important implications for the understanding of the various functions of NS2 and the sequential events of the viral life cycle.

CONCLUSIONS

Much work is still required in the study of the NS2/3 protease. Although several studies over the past decade have focussed on NS2/3 cleavage, the catalytic mechanism of the enzyme remains controversial. Initial attempts at characterizing the enzyme were limited to *in vitro* and cell expression systems and despite the development in recent years of *in vitro* systems in which the processing reaction can be studied using purified recombinant proteins, a definitive classification has not yet been determined. A three dimensional structure of NS2/3 is very much needed and will likely yield important insights into the mechanism of action of the enzyme.

Similarly, a robust cell culture system for the study of the viral life cycle is of urgent need (see Chapter 16). Such a system will be crucial to precisely define the roles of NS2/3 cleavage and the NS2 protein in the complete viral life cycle. Of particular interest are the observations that NS2 could potentially modulate the host cell environment during HCV infection through interference with gene expression and cellular apoptosis. However, it will be necessary to validate these findings in a more physiologically relevant setting.

Although its mode of action is unclear, NS2/3 cleavage is absolutely required for persistent viral infection in a chimpanzee. The HCV NS2/3 protease shares no obvious sequence homology to any known proteases in the animal kingdom and

would therefore make an attractive target for antiviral therapy. The elucidation of the crystal structure of NS2/3, its mechanism of action and precise functions in replication will help to generate important information for the development of strategies for inhibition of NS2/3 processing, which could become the basis for novel HCV therapies in the future.

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