The Hepatitis C Virus NS2/3 Protease

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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. Based on the recently elucidated crystal structure of NS2, NS2/3 has been proposed to function as a cysteine protease despite its lack of sequence homology to proteases of known function. In addition, although shown to be required for HCV genome replication and persistent infection in a chimpanzee, the role of NS2/3 cleavage in the viral life cycle has not yet been fully investigated. 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 hepatitis C virus (HCV) is a serious health concern and an important cause of chronic liver disease, leading to cirrhosis and hepatocellular carcinoma in humans (Choo *et al*., 1989; Hoofnagle, 1997; Kuo *et al*., 1989). It is estimated that nearly 200 million individuals worldwide are currently infected with HCV. Of particular concern is that the virus establishes a chronic infection in ~85% of cases and that there are no specific and broadly effective anti-HCV compounds to date (Hoofnagle, 1997). Belonging to the *Flaviviridae* family, HCV is a single-stranded RNA virus of positive polarity that encodes a single polypeptide chain that is cleaved co- and post-translationally into both structural (core, E1, E2, p7) and non- structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins (reviewed in Reed and Rice, 2000).

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 (Fig. 1). The amino terminus of NS2 is cleaved from the adjacent p7 protein by host signal peptidases in a membrane-dependent 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. Many studies have focused on the residues and sequences required for efficient NS2/3 processing and it was recently established

to function as a novel cysteine protease. 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 review 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). Figure 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 on 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 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 any of these amino acids to alanine completely inhibits NS2/3 cleavage activity (Grakoui *et al*., 1993; Hijikata *et al*., 1993a; Welbourn *et al*., 2005). 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
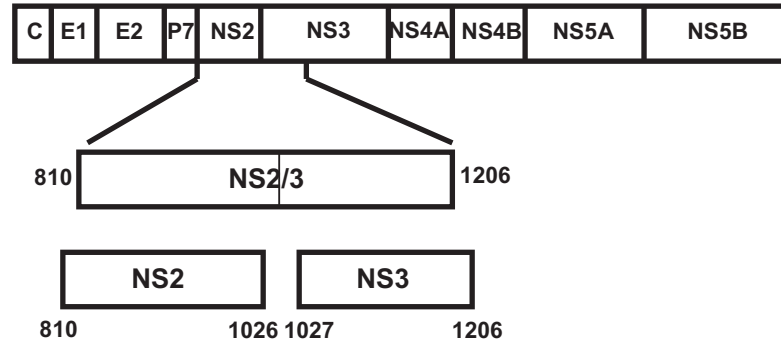
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Fig. 1. The HCV NS2/3 protease. The NS2/3 protease is shown in the context of the HCV polyprotein. NS2 and the protease domain of NS3 (from aa 810 to 1206) constitute NS2/3, which undergoes autocatalytic cleavage between aa 1026 and 1027.

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, WRLL↓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. have demonstrated the requirement for the ATP hydrolyzing ability of molecular chaperone HSP90 for efficient cleavage in in vitro and cell based assays (Waxman et al., 2001). 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: a novel cysteine protease

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 then proposed NS2/3 may be a novel cysteine protease with a catalytic dyad composed 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 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 was relieved by the addition of $ZnCl_2$, $CdCl_2$ or $MgCl_2$. 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 showed strong inhibition of NS2/3 processing, no single cysteine was found to be more susceptible to these alkylating agents (Pallaoro et al.,
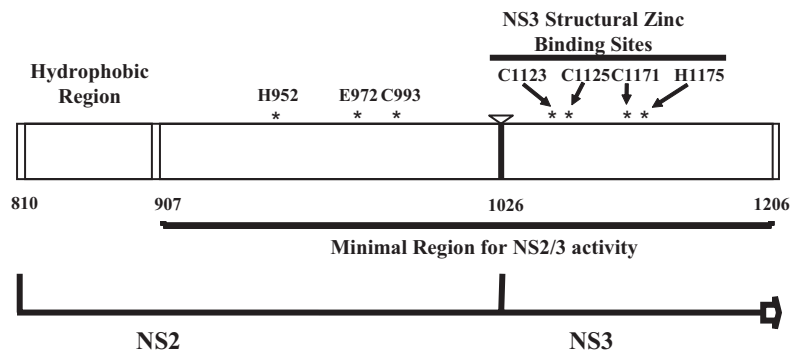

Fig. 2. Functional domains of the NS2/3 protease. The NS2/3 protease encompasses an N-terminal hydrophobic region, with a minimal domain required for activity between aa 907 and 1206. Residues in NS2 required for NS2/3 processing (H942, E972, C993), as well as residues in NS3 responsible for the coordination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

2001) and it was only with the recently elucidated crystal structure of its NS2 portion that the enzyme was firmly established as a novel cysteine protease (Lorenz *et al*., 2006).

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).

The crystal structure of the N-terminal truncated NS2 portion (aa 904–1026) of the NS2/3 enzyme was recently solved (Lorenz *et al*., 2006). This structure shows a dimeric enzyme containing two composite active sites. Each active site shows the catalytic histidine and glutamic acid residues present on one monomer with the catalytic cysteine of the triad contributed by the other monomer. As this structure essentially depicts the NS2 cleavage product of the NS2/3 enzyme, it is interesting to note that the C-terminal residues of the protein remain coordinated in the active sites, which was suggested to potentially function to inactivate the protease after cleavage into NS2 and NS3 has occurred.

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* was 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 neighboring polyprotein chains. These observations were recently confirmed by the dimeric crystal structure of NS2 (Lorenz *et al*., 2006). In addition, co-transfection in mammalian cells of mutant NS2/3 constructs in which either the catalytic cysteine or histidine residues are mutated to alanine, still results in some cleavage of the NS2/3 precursor due to the presence of one functional active site on the dimer (contributed by the cysteine residue on the histidine-mutant monomer and the histidine residue of the cysteine-mutant monomer) (Lorenz *et al*., 2006). As NS2/3 cleavage was widely believed to be an intramolecular event, the significance of these recent findings with respect to the polyprotein

processing events of HCV infection will be of interest to determine.

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). In addition, efficient NS2/3 cleavage is required for replication of the viral RNA genome in a NS2–3-′UTR replicon system (Welbourn *et al*., 2005). However, NS3–3-′UTR subgenomic replicons not encoding the NS2 protein replicate efficiently in Huh-7 cells (Lohmann *et al*., 1999), suggesting NS2 itself 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 stains 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. It has been shown in the replicon system that mutant, uncleaved NS2/3 is rapidly degraded in cells (Welbourn *et al*., 2005), which, should regulation of HCV NS2/3 cleavage occur, could potentially be a way for the virus to regulate the levels of the NS3 protein and thus its replication. 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, as an infectious cell culture system to study the HCV life cycle has only recently been established (Lindenbach *et al*., 2005, Wakita *et al*., 2005, Zhong *et al*., 2005), studies focusing on the replication complex have so far mainly

used the subgenomic replicon system, 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). One group has also directly shown an interaction between NS2 and NS3 using co-immunoprecipitation experiments (Kiiver *et al*., 2006). 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.

Interestingly, the recent construction of intergenotypic infectious chimeras containing Con1 (gentotype 1b), H77 (1a) or J6 (2a) structural proteins in the JFH-1 (2a) backbone, suggests that the optimal junction point of the chimera is within NS2, immediately following the first transmembrane domain (Pietschmann *et al*., 2006). The most efficient chimeras contained the C-term of NS2 of the same strain as the replicase and the N-term of the same strain as the structural proteins. It is therefore suggested that NS2 may play a dual role in the viral life cycle and in addition to its role in NS2/3 cleavage, may also be involved in virus assembly and release, possibly through association of the N-terminus of NS2 with structural proteins and/or p7. However, the exact mechanisms with which NS2 may be involved in virus assembly and release remain to be determined.

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. 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, Kaukinen *et al*. (2006) have also shown NS2 to inhibit a variety of cellular promoters involved in cytokine gene expression, such as INF-β, CCL5/RANTES and CXCL10/IP-10 promoters (Kaukinen *et al*., 2006). Several other HCV-encoded proteins, including core, NS3/4A, NS4B and NS5A, have also been demonstrated to alter cellular gene expression though a variety of mechanisms (Kato *et al*., 1997; Kato *et al*., 2000; Naganuma *et al*., 2000; Ray *et al*., 1995; Kaukinen *et al*., 2006). 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 signaling 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.

Furthermore, expression of the NS2 protein has been shown to inhibit cell proliferation in both transient (Huh-7) and stable (Vero, HeLa) cell assays (Yang *et al*., 2006). Expression of NS2 was found to be associated with a 40–50% decrease in the growth rate of the cells, resulting from cell cycle arrest in the S-phase. This was also correlated with a decrease in the levels of cyclin A RNA and protein. Although the mechanisms involved in these observations are currently unknown, it is suggested that NS2 may modify the cell cycle to help provide a cellular environment that is advantageous for viral replication (Yang *et al*., 2006).

The roles of mature cleaved NS2 remain largely unexplored. Although some possible functions have been proposed and are described here, the use of the newly discovered JFH-1 cell culture system will hopefully help 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 focused on NS2/3 cleavage, the catalytic mechanism of the enzyme has only recently been established. The three dimensional structure of NS2 provides many insights into the mechanism of action of the enzyme as well as raises interesting questions as to the polyprotein processing events, however it still only represents the post-cleavage form of the protein. How the NS3 portion of the enzyme, and potentially the rest of the yet uncleaved non-structural polyprotein, fits into this model remains an interesting and much more challenging question.

Similarly, a robust cell culture system for the study of the viral life cycle has finally been established (Lindenbach *et al*., 2005, Wakita *et al*., 2005, Zhong *et al*., 2005). 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 now be necessary to validate these findings in this more physiologically relevant setting.

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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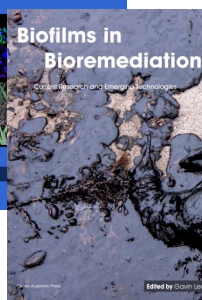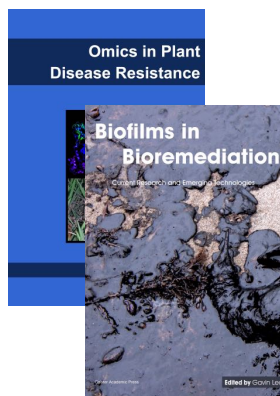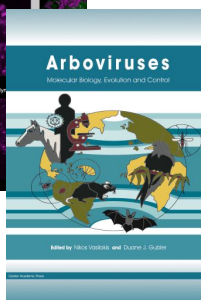
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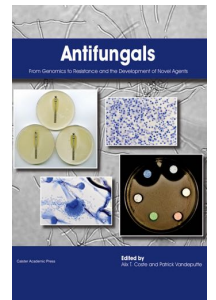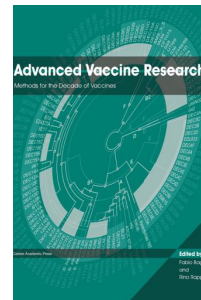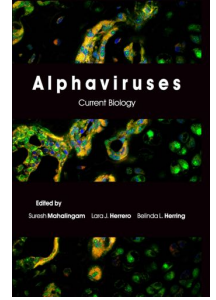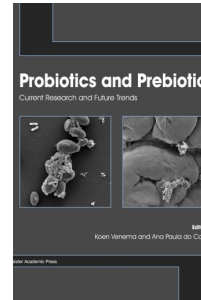
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