Internal Processing of Hepatitis C Virus NS3 Protein

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Hepatitis C virus (HCV) NS3 protein contains at least three enzymatic activities: NS2-3 protease, NS3 serine protease, and NTPase/RNA helicase. It has been shown that NS2-3 cleavage is mediated by NS2-3 protease, whereas NS3 serine protease is responsible for the other four cleavage sites of the nonstructural (NS) region. In this study, we showed that the internal cleavage of NS3 protein produced two products of 49 kDa (NS3a) and 23 kDa (NS3b) when the entire NS3 region (aa 1027-1657) or the whole open reading frame (aa 1-3010) was expressed in mammalian and insect cells. By means of site-directed mutagenesis, we demonstrated that NS3a/NS3b cleavage occurs within the RNA helicase sequence motif that is highly conserved in the Flaviviridae family and that neither NS2-3 protease nor NS3 serine protease was responsible for this cleavage. The NS3 protease of flaviviruses, dengue virus type 2, for example, has been shown to mediate the internal cleavage of NS3. The NS3 proteins of HCV and dengue virus may thus be cleaved internally at the same sequence by different mechanisms of proteolysis. Also discussed is a possible role for the internal processing of HCV NS3 in the viral life cycle and its pathogenesis. © 1999 Academic Press

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of posttransfusion and sporadic non-A, non-B hepatitis (Choo et al., 1989; Kuo et al., 1989). The majority of HCV-infected individuals develop chronic hepatitis. This frequently leads to liver cirrhosis and hepatocellular carcinoma (Saito et al., 1990).

HCV is a positive-stranded RNA virus that belongs to the Flaviviridae family (Choo et al., 1989). Its 9.5-kb genome contains a large open reading frame that encodes at least 10 viral proteins in the order NH2-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. The polyprotein is then processed by host signalase and viral proteases. Processing at the core/E1, E1/E2, E2/p7, and p7/NS2 junctions is mediated by a host signal peptidase (Hijikata et al., 1991; Lin et al., 1994; Mizushima et al., 1994). Processing at the NS2/NS3 junction is an intermolecular cleavage that requires the C-terminal domain of NS2 and the N-terminal domain of NS3 protein (Hijikata et al., 1993; Grakoui et al., 1993b). The viral serine protease located in the N-terminal domain of NS3 cleaves the remaining junctions in the nonstructural region of the polyprotein, including the cis-cleavage at the NS3/NS4A junction and the trans-cleavage at the NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B junctions. The C-terminal domain of NS3 has both NTPase and helicase activities (Kim et al., 1996). The NS3 protein is a multifunctional protein, required for viral polyprotein processing and virus replication. The crystal structures of HCV NS3 serine protease and helicase revealed novel features, including a structural zinc-binding site and a long N-terminus that interacts with neighboring molecules by binding to a hydrophobic surface patch (Love et al., 1996; Kim et al., 1996; Yao et al., 1997). It is of interest to study how the NS3 protein determines its multiple function.

In this study, we have found that the NS3 protein was internally cleaved within an RNA helicase motif and have analyzed the mechanism of this processing.

RESULTS

Internal cleavage of the NS3 protein in insect cells

To express the entire open reading frame (ORF) of HCV, we utilized the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) expression system. Tn5 cells were infected with Ac394 (Aizaki et al., in preparation) (Fig. 1), a recombinant baculovirus expressing a full-length HCV ORF (aa 1 to 3010). We used wild-type baculovirus AcNPV as a negative control. By Western blotting with specific polyclonal antibodies and monoclonal antibodies, properly processed proteins from core to NS5B proteins were detected in the cells infected with Ac394 (Aizaki et al., in preparation). Proteins of 72 and 49 kDa were immunoprecipitiated by an anti-NS2/NS3 rabbit polyclonal antiserum directed against the C-terminal portion of NS2 and the N-terminal...
third of NS3 (aa 990 to 1243) (Fig. 2A). The 72-kDa protein is the full-size NS3. The 49-kDa protein is predicted to be an N-terminal portion of the further processed NS3 proteins and is designated NS3a.

To analyze further processing of the NS3 protein, Tn5 cells were infected with AcNS3 (Fig. 1), a recombinant baculovirus expressing aa 1027 to 1657, which encompassed the entire NS3 region. The infected cells were analyzed again by Western blotting with the anti-NS2/NS3 and anti-NS3C polyclonal antibodies. The anti-NS3C
polyclonal antibody was raised against the C-terminal portion of the NS3 protein spanning from aa 1565 to 1657. The 72- and 49-kDa proteins were detected with anti-NS2/NS3 antibody (Fig. 2B). On the other hand, 72- and 23-kDa proteins were detected by the anti-NS3C antibody (Fig. 2C). Therefore, the 23-kDa protein was considered to be a C-terminal portion of the further processed NS3 protein and was designated NS3b. These results also indicated that the majority of the expressed NS3 protein remained uncleaved under the conditions used.

Sequence alignment of the putative cleavage site between NS3a and NS3b

The above immunoblot analyses using N-terminal- and C-terminal-specific antibodies against NS3 suggest that the further cleavage site between NS3a and NS3b must exist within the C-terminal region of NS3. HCV NS3 RNA helicase belongs to the DEAD box NTPase/RNA helicase family (Choo et al., 1991; Gorbalenya et al., 1989). The DEAD box protein family has eight highly conserved motifs and consists of three subfamilies, DEAD proteins, DEAH proteins, and DEXH proteins. The HCV NS3 protein has a sequence motif of DECH, which belongs to the DEXH protein subfamily. The HCV NS3 protein has sequence motifs including XXXXXGKS, DECH, TAT, XXGXX, and QRRGRTGR.

Interestingly, similar processed forms of NS3 were detected in tick-borne encephalitis virus (TBE)-infected cells and also in Dengue virus type 2 (DEN 2)-infected cells (Falgout et al., 1991; Pugachev et al., 1992). It was shown that the internal cleavage of the DEN 2 NS3 protein occurs at QRR1933/GR within its RNA helicase motif (Keng and Wright, 1997). We compared the C-terminal region of NS3s of HCV, flaviviruses, and pestiviruses. As shown in Fig. 3, this final RNA helicase motif is completely conserved among all the HCV genotypes (1a to 6b). It is also notable that this sequence is highly conserved in the Flaviviridae family. We thus predicted that the HCV NS3 protein may be cleaved into NS3a and NS3b at the QRR1488/GRTGR site as was shown in DEN 2-infected cells (Fig. 3, arrow).

Mutational analyses of the putative NS3a/NS3b site in insect cells

Using site-directed mutagenesis, we determined that HCV NS3 is cleaved at the putative NS3a/NS3b site, QRR1488/GRTGR. We constructed two mutant baculoviruses, AcNS31488A, possessing a single amino acid mutation in the putative cleavage site (Arg1488 to Ala), and AcNS31496A, a negative control (Arg1496 to Ala) (Fig. 1). In Trn5 cells infected with AcNS3 or AcNS31496A, full-size NS3, NS3a, and NS3b were detected (Fig. 4, lanes 2 and 4). In contrast, neither NS3a nor NS3b was detected in the cells infected with AcNS31488A (Fig. 4, lane 3). These data strongly suggest that the NS3 protein is cleaved into NS3a and NS3b at the QRR1488/GRTGR site.

Internal cleavage of the NS3 protein in mammalian cells

To confirm that the NS3 protein is cleaved at the same site in mammalian cells as in insect cells, we constructed expression plasmids pCAGNS3 and pCAGNS31488A. 293T cells were transfected with pCAGNS3 or pCAGNS31488A. As a negative control, cells were transfected with a vector plasmid without insertion, pCAG(BglII) (Aizaki et al., in preparation). In 293T cells transfected with pCAGNS3, full-size NS3, NS3a, and NS3b were detected (Fig. 5, lane 2). In contrast, neither NS3a nor NS3b was detected in 293T cells transfected with pCAGNS31488A, indicating that mutation of Arg1488 to Ala abolished the cleavage at the putative NS3a/NS3b site (Fig. 5, lane 3).

These results indicated that the NS3 protein was cleaved at
the QRR1488/GRTGR site in mammalian cells as well as in insect cells. Without proper quantitative analysis, it is not clear whether the level of cleavage in mammalian cells is the same as in insect cells.

Pulse–chase analysis demonstrated that NS3a was produced soon after the 20-min labeling and that the ratio of NS3a to NS3 did not change during the 60-min chase period (data not shown). It is thus possible that a constant amount of NS3 is cotranslationally processed into NS3a and NS3b.

Analysis of mechanism for the internal cleavage of the NS3 protein

We then tested whether cellular protease(s) or HCV protease(s) is involved in the further processing of the NS3 described above. To determine the possibility of HCV proteases' role, we introduced site-directed mutations into NS2-3 and NS3 serine proteases. A recombinant baculovirus, Ac2952, carried almost all the NS2/NS3 region (aa 848 to 1652) (Fig. 1). To inactivate the NS3 serine protease, aa 1083, histidine, was converted to alanine. This mutant baculovirus was designated Ac2952H1083A (Figs. 1 and 6A). To inactivate the NS2-3 protease activity, aa 993, cysteine, was converted to alanine. This mutant baculovirus was designated Ac2952C993A (Figs. 1 and 6A). We confirmed that Ac2952 expressed NS3 serine protease activity, whereas Ac2952H1083A expressed an inactive NS3 serine protease by using NS5A/NS5B regions as a substrate (data not shown). In the cells infected with Ac2952C993A, the

![Image](image1.png)

FIG. 4. Immunoblot analysis of the internal cleavage of NS3 in insect cells. Tn5 cells infected with AcNS3, AcNS3R1488A, or AcNS3R1496A were harvested at 60 h postinfection and analyzed by immunoblot analysis by using anti-NS2/NS3 polyclonal antibody (A) or anti-NS3C polyclonal antibody (B). The full-size NS3 protein (NS3), processed products NS3a detected by anti-NS2/NS3 antibody, and NS3b detected by anti-NS3C antibody are indicated by arrows. Lanes 1, AcNPV-infected cells; lanes 2, AcNS3-infected cells; lanes 3, AcNS3R1488A-infected cells; lanes 4, AcNS3R1496A-infected cells. The numbers on the left indicate molecular sizes (kDa).

![Image](image2.png)

FIG. 5. Immunoprecipitation analysis of the internal cleavage of NS3 in mammalian cells. 293T cells transfected with pCAGBgIII, pCAGNS3, or pCAGNS3R1488A were labeled with Tran 35S-label and immunoprecipitated with anti-NS2/NS3 polyclonal antibody (A) or anti-NS3C polyclonal antibody (B). The immunoprecipitates were subjected to SDS–PAGE and autoradiography. Lanes 1, pCAG(BgIII); lanes 2, pCAGNS3; lanes 3, pCAGNS3R1488A. The numbers on the left indicate molecular sizes (kDa). The full-size NS3 protein (NS3), processed products NS3a detected by anti-NS2/NS3 antibody, and NS3b detected by anti-NS3C antibody are indicated by arrows.
processing between the NS2/NS3 junction did not occur, nor did we detect the resulting C-terminal truncated NS2 protein (NS2ΔN) (Fig. 6B, lane 4). This indicated that the NS2-3 protease expressed by Ac2952C993A was inactive and does not cleave the NS2/3 junction.

As shown in Fig. 6B (lanes 2 and 3), the NS3a protein was detected in the cells infected with Ac2952 or Ac2952H1083A, indicating that the internal cleavage of the NS3 protein occurs even when the NS3 serine protease is inactive. The 68-kDa protein, which was observed in the cells infected with Ac2952C993A (Fig. 6B, lane 4), is considered to be produced by processing the 2952 protein at the NS3a/NS3b site, since the inactivated NS2-3 protease did not cleave at the NS2/NS3 site in the cells infected with Ac2952C993A. NS3b protein truncated by five amino acids at the C-terminal (NS3bΔC) was detected in the cells infected with Ac2952, Ac2952H1083A, or Ac2952C993A, irrespective of activity of the viral proteases (Fig. 6C, lanes 2, 3, and 4). From these data, we have concluded that the HCV proteases are not responsible for the cleavage at the NS3a/NS3b site and that this cleavage is probably mediated by a cellular protease.

**DISCUSSION**

Further processing of the NS3 protein was investigated by transient expression in both mammalian and insect cells to determine the internal cleavage site within the NS3 protein and to examine the mechanism of its processing. We found that the NS3 protein was cleaved into NS3a and NS3b in both insect and mammalian cells. Further studies using site-directed mutants revealed that the NS3 protein was specifically processed at the QRR1488/GRTGR site within the RNA helicase motif. Even when the NS2-3 protease and NS3 serine protease are inactive, internal cleavage of the NS3 protein occurs, suggesting that HCV proteases are not responsible for the internal cleavage and that this cleavage is probably mediated by cellular protease.

We observed that the NS3 protein was processed at the QRR1488/GRTGR site in both insect and mammalian cells, indicating that the NS3 protein is internally processed very specifically. Specific cleavage of the NS3 protein at the QRR1488/GRTGR site is considered to occur in HCV-infected cells.

The functional role of the internal cleavage within the NS3 protein is not known. However, it is of interest that internal cleavage within the NS3 protein was also reported in cells infected with DEN-2 and TBE (Pugachev et al., 1992; Arias et al., 1993). It has been shown that production of NS3a for DEN-2 resulted from cleavage at the QRR/GR site within an RNA helicase sequence motif (Keng and Wright, 1997). Our data demonstrated that the internal cleavage within the NS3 protein of HCV also...
occurred within the RNA helicase motif. The internal cleavage within the NS3 protein may be a common phenomenon among the Flaviviridae family and may play an important role in their life cycle. This motif is proposed to have a role in ATP hydrolysis and RNA binding. It was shown that Arg1488 was important for RNA binding and RNA helicase activity (Kim et al., 1997). The crystal structure of the HCV RNA helicase domain revealed that the conserved QRRGRTGR sequence spans the C-terminus of a short α-helix and the first segment of a more extended loop (Yao et al., 1997). Residues required for RNA winding are localized on the C-terminal region. Internal cleavage within the NS3 protein could consequently inactivate the RNA-dependent RNA helicase, providing a mechanism for regulation of viral RNA synthesis.

Arias et al. (1993) proposed that NS3 was further cleaved by NS3 serine protease derived from DEN-2. In contrast, our data demonstrated that HCV proteases are not responsible for the internal cleavage. The cleavage sites recognized by a trypsin-like serine protease are usually characterized by two basic amino acids followed by a residue with a short side chain (Arg, Ala, or Gly). The following amino acid is also a basic residue, either Lys or Arg. At the sites cleaved by flavivirus serine proteases, only the P2, P1, and P1' are highly conserved among all flaviviruses. Basic residues (Arg or Lys) are usually found at the P2 and P1 positions, with the exception of Glu at the P2 position of the 2B/3 site of the four dengue virus serotypes. Our proposed internal cleavage site within NS3 is consistent with this sequence. The N-terminal domain of NS3 derived from HCV has been found to contain the catalytic motif of a trypsin-like serine protease (Miller and Purcell, 1990). The positions of His1083, Asp1107, and Ser1165 are strictly conserved among all HCVs. Their relative order and spacing in the sequence correspond to the catalytic triad of the trypsin family. However, the NS3 serine protease exhibits highly unusual features for a trypsin-like protease. Cleavage specificity of this enzyme is distinct from that of the flavivirus enzymes. Polar residues (Cys or Thr) are found at the P1 position, and acidic residues (Glu or Asp) are found at the P6 position (Grakoui et al., 1993a). Similar to the flavivirus enzymes, residues with small side chains, Ser or Ala, seem to be preferred at the P1' position. These observations support the conclusion that HCV NS3 serine protease is not responsible for the cleavage at the QRR1488/GRTGR site. Therefore, the internal cleavage in the NS3 protein is probably mediated by cellular protease.

It is unknown whether the internal cleavage within NS3 affects the NS3 serine protease activity. The NS3a protein is presumably an active protease, because proteolysis has been determined in a number of expression systems containing truncated forms of NS3 (Hijikata et al., 1991; Grakoui et al., 1993a; Tomei et al., 1993; Suzuki et al., 1995; Shoji et al., 1995). Little is known about the pathogenic effects of the HCV NS3 protein on host cells. Borowski et al. (1996) have shown that 1487RRGRT-GRGRRGITR in the HCV NS3 region has a sequence highly similar to the inhibitory site of the heat-stable inhibitor of cAMP-dependent protein kinase (PKA) and to the autophosphorylation site in the hinge region of the PKA type II regulatory domain. It has been shown that bacterially expressed HCV NS3 protein containing amino acids 1189 to 1525 can bind to the catalytic subunit of PKA and inhibit the translocation of the C-subunit into the nucleus (Borowski et al., 1997). Pathogenic effects of a number of viruses result from the disturbance of intracellular signal transduction cascades caused by viral antigens. Our data suggest that the internal cleavage at the NS3a/NS3b site could reveal this sequence, which has the potential to inhibit PKA function. It should be of interest to determine whether production of the NS3b protein affects the PKA function and cause pathogenic effects on host cells. Recently infectious cDNA clones of HCV have been established (Kolykhalov et al., 1997; Yanagi et al., 1998). It is important to study the biological functions and pathogenic effects of the internally cleaved HCV NS3 protein by mutation analyses at the NS3a/NS3b site of the infectious cDNA clones.

MATERIALS AND METHODS

Cells and viruses

Recombinant baculoviruses were constructed by use of the transfer vector pAcYM1 (Matsuura et al., 1987). AcNPV and recombinant baculoviruses were grown and assayed in Spodoptera frugiperda (SI) 9 cells in TC100 medium (Gibco Laboratories, Grand Island, NY) supplemented with 0.26% bacterotryptose broth (Difco, Detroit, MI), 100 mg of kanamycin/ml, and 10% fetal bovine serum. Recombinant baculoviruses were isolated by co-transfection with infectious viral DNA as described previously (Matsuura et al., 1987). Recombinant baculoviruses were amplified in SI9 cells and infectious titers were determined by plaque assay. Trichoplusia ni (Tn) 5 cells were grown in Ex-cell 405 (BSC Biosciences, Lenexa, KS). 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing 2 mmol of L-glutamine, penicillin (50 IU/ml), streptomycin (50 mg/ml), and 10% fetal bovine serum.

Construction of plasmids

All expression plasmids were derivatives of pET21b (Novagen, Madison, WI), pCAG(BgIII) (Niwa et al., 1991; Aizaki et al., in preparation), pAcYM1 (Matsuura et al., 1987), and pGEX-4T-1 (Pharmacia Biotech, Uppsala, Sweden). Type 1b HCV cDNA clone, HCV NIH-J1, was derived from a blood sample of a healthy HCV carrier (Kubo et al., 1990; Takeuchi et al., 1990; Aizaki et al., 1998). Transfer vector pcdAc934 encodes an entire ORF...
of the HCV genome from nt 341 to 9370 (aa 1 to 3010) (Aizaki et al., 1998; Aizaki et al., in preparation). pETNS3 was derived from polymerase chain reaction (PCR) amplification of nt 3418 to 5311 (aa 1027 to 1657) with sequence-specific primers, using plasmid pNIHJ1 (Aizaki et al., 1998) as the template. The resultant pETNS3 was expected to express the full-size NS3 protein. Specific primers were as follows: sense primer, 5’-AGGATATCAT-GGCCGCCATCACGGC-3’; antisense primer, 5’-AACTCGAGAGTGACGACCTTATAGGT-3’. All constructs were confirmed by sequencing analysis after PCR. An EcoRV and XhoI fragment of the amplified DNA fragment was cloned into the expression vector of pET21b, which had been cleaved with NdeI, blunted with Klenow polymerase, and digested with XhoI. Site-directed mutant pETNS3R1488A (Arg1488 to Ala), possessing a single amino acid mutation in the putative cleavage site, and a negative control pETNS3R1496A (Arg1496 to Ala) were obtained by inserting mutations in PCR primers. The substitution mutation of Arg to Ala was made by site-directed mutagenesis according to the method described by Ito et al. (1991). The specific primers used to introduce the mutation were as follows: R1488A, 5’-GTCCTACCGGCCCATCACGGC-3’, R1496A, 5’-TAGATGCTGACCTTATAGGT-3’. To construct transfer vector pAcNS3, a XbaI–Bpu1102I fragment derived from pETNS3 after treatment with Klenow polymerase was cloned into the Smal site of pAcYM1. pAcNS3R1488A and pAcNS3R1496A were constructed similarly. pCAGNS3 was obtained by cloning into the BglII site of pCAGBgIII the XbaI–Bpu1102I fragment derived from pETNS3 after treatment of both DNA fragments with Klenow polymerase. pCAGNS3R1488A was constructed similarly. pAc2952 encodes the HCV sequence from aa 847 to 1657 (Matsuura et al., 1994). 293T cells (2 × 10^5 cells in a 35-mm plate) were transfected with expression plasmids incubated for 24 h. Each dish was radiolabeled with 20 μCi of Tran^{35}S-label for 4 h after 2 h of starvation with medium deficient in FBS, methionine, and cysteine. Cells were washed twice with 500 μl of phosphate-buffered saline, dissolved in 400 μl of TNE buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10 μg of aprotinin/ml). Tn5 cells were infected with the recombinant baculovirus at a multiplicity of 5 PFU/cell in 35-mm tissue culture dishes and incubated at 26.5°C for 24 h. Each dish was radiolabeled with 20 μCi of Tran^{35}S-label for 4 h after 2 h of starvation with TC-100 medium deficient in FBS, methionine, and cysteine. Prior to immunoprecipitation, SDS...
and dithiothreitol were added to the cell lysates to final concentrations of 2% and 10 mM, respectively. Cell lysate (100 μl) was suspended in 900 μl of TNE buffer and incubated with 0.5 ml of anti-NS2/NS3 or anti-NS3C polyclonal antibody and 20 ml of Protein A–Sepharose (Pharmacia) (50% suspension (v/v) in TNE buffer) for 1 h at 4°C with rotating. After centrifugation at 8000 g for 10 s at 4°C, pellets were washed twice with TNE buffer. The immunoprecipitates were analyzed by SDS–PAGE.

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