The NS5A Protein of the Hepatitis C Virus Genotype 1a Is Cleaved by Caspases to Produce C-terminal-truncated Forms of the Protein That Reside Mainly in the Cytosol*

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The nonstructural 5A (NS5A) protein of the hepatitis C virus (HCV) is a multifunctional protein that is implicated in viral replication and pathogenesis. We report here that NS5A of HCV-1a is cleaved at multiple sites by caspase proteases in transfected cells. Two cleavage sites at positions Asp154 and 248D are cleaved at multiple sites by caspase proteases in transfected cells. Cleavage at Asp154 has been previously recognized as one of the caspase cleavage sites for the NS5A protein of HCV genotype 1b (1, 2) and results in the production of a 17-kDa fragment. The sequence 248DXXD is a novel caspase recognition motif for NS5A and is responsible for the production of a 31-kDa fragment. Furthermore, we show that Arg217 is implicated in the production of the previously described 24-kDa product, whose accumulation is affected by both calpain and caspase inhibitors. We also showed that caspase-mediated cleavage occurs in the absence of exogenous proapoptotic stimuli and is not related to the accumulation of the protein in the endoplasmic reticulum. Interestingly, our data indicate that NS5A is targeted by at least two different caspases and suggest that caspase 6 is implicated in the production of the 17-kDa fragment. Most importantly, we report that, all the detectable NS5A fragments following caspase-mediated cleavage are C-terminal-truncated forms of NS5A and are mainly localized in the cytosol. Thus, in sharp contrast to the current view we found no evidence supporting a role for caspase-mediated cleavage in the transport of the NS5A protein to the nucleus, which could lead to transcriptional activation.

Caspases are cysteine-dependent proteases that constitute the central executioners of apoptosis (3–4). Caspases are expressed as proenzymes, and they become activated through cleavage at internal aspartic acid residues by other caspases (4–5). Procaspases with a long N-terminal prodomain, called initiator caspases (caspase 2, 8, 9, and 10), are activated first and then cleave and activate procaspases with a shorter N-terminal prodomain, called executioner caspases (caspase 3, 6, and 7) (4). The activated executioner caspases then cleave a number of target proteins, important for several cellular functions, leading to the ultimate destruction of the cell (6). Up to date, 14 mammalian caspases have been identified that are implicated in different aspects of cell death, but the exact function of each individual caspase is still largely unknown (4).

Surprisingly, however, growing evidence now indicates a participation of caspases and other apoptotic regulators in nonapoptotic cellular processes such as cell cycle control, cell differentiation, and inflammation (7–9). Most interestingly, caspases are also utilized by a number of viruses for cleavage of their own proteins (10). Several caspases have been shown to target the structural proteins of different viruses, including transmissible gastroenteritis coronavirus (TGEV) (11), human astrovirus (HAstv) (12), influenza A virus (13), and feline calcivirus (FCV) (14), as well as nonstructural viral proteins, such as the NS1 from the Aleutian mink disease parvovirus (ADV) (15), the adenovirus (Ad) E1A (16), or the immediately early protein 22 from herpes simplex virus (HSV-1) (17), with an impact on viral pathogenesis. Interestingly, caspase 3, the executioner caspase that targets the majority of cellular substrates during apoptosis, is also responsible for cleaving many viral proteins, and numerous studies have implicated caspase 6, 7, and 2 in the cleavage of different viral protein substrates (11, 14, 16). Because most viruses encode inhibitors of caspases to evade cellular antiviral mechanisms that lead cells to apoptosis, (18–21) it is not immediately apparent why viruses rely on caspases for cleavage of their own proteins. On the other hand, some viruses induce apoptosis, resulting in virus dissemination, whereas certain viruses do both at different stages in their propagation (10, 19). Thus, the cumulative data on the novel requirement of caspase activity for virus propagation combined with the growing evidence for the participation of caspases in several nonapoptotic cellular processes, has triggered the hypothesis that caspase activation may represent an important point of control for virus replication that remains to be explored (10).

Hepatitis C virus (HCV), a small hepatotropic RNA virus, affects ~3% of the population worldwide, leading to major health problems. HCV infection is associated with high rates of progression to chronic infection, which often lead to liver cirrhosis and hepatocellular carcinoma with fatal outcome (22–24). Currently, no vaccine against HCV is available. The hepatitis C virus is classified within the Hepacivirus genus of Flaviviridae family (25). The viral genome consists of a 9.6-kb, single-stranded, positive-sense RNA molecule, which encodes a precursor polyprotein of about 3,000 amino acids. Proteolytic processing of the polyprotein by host and viral proteases yields at least 10 mature viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (26). An additional protein known as F or ARFP or core+1 was previously described (27). The 5’- and the 3’-untranslated regions of the viral genome are highly conserved and contain control elements for viral replication, translation of the viral polyprotein (26).

The HCV NS5A protein is a multifunctional serine phosphoprotein encoded by an alternative reading frame within the core coding region, but its function remains unknown (27–29). The 5’- and the 3’-untranslated regions of the viral genome are highly conserved and contain control elements for viral replication and translation of the viral polyprotein (26).

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2 The abbreviations used are: HCV, hepatitis C virus; HCMV, human cytomegalovirus; GFP, green fluorescent protein; nt, nucleotide; CHX, cycloheximide; PBS, phosphate-buffered saline; CHAPS, 3-(3-Cholamidopropyl)dimethylammonio)1-propanesulfonic acid; Z, benzoyloxycarbonyl; FMK, fluoromethylketone; ER, endoplasmic reticulum; TNF, tumor necrosis factor; NLS, nuclear localization factor; aa, amino acids; wt, wild type; p.t., post-transfection.
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of 56–58 kDa in size (30–32), that participates in viral replication, translation, and HCV-mediated pathogenesis (33–36). As with most of the nonstructural viral proteins, NS5A participates in the formation of the viral replication complex, on the cytoplasmic side of the ER, where it can also directly interact with the RNA-dependent RNA polymerase of the virus (37–39). An amphipathic α-helix located in the N-terminal region of NS5A is responsible for its anchorage on the ER membranes despite the presence of a functional NLS in the C-terminal part of the protein (40–41). Thus, recombinant N-terminal-deleted forms of NS5A, which lack the membrane anchoring signal, are almost exclusively localized to the nucleus and exhibit transactivation properties (42–43).

Furthermore, NS5A interacts with a number of cellular proteins implicated in the interferon-mediated antiviral response (44–47), transcription (42–43, 48–51), apoptosis (52–57), cell growth, and differentiation (58–59), lipid metabolism (60), and signal transduction (38, 45, 47, 61), denoting the potential of the protein to affect the host environment. NS5A also perturbs Ca²⁺ homeostasis, induces oxidative stress and activates the Ca²⁺-dependent calpain proteases (62–64). Interestingly, it was recently shown by our laboratory that calpains cleave NS5A to produce shorter N-terminal forms of the protein, suggesting that in addition to phosphorylation, calpain cleavage may modulate the numerous activities of the NS5A protein (65).

Additionally, it was recently shown that under certain conditions, caspase-like proteases can cleave NS5A of HCV-1b into a few fragments, producing N- and C-terminal-truncated forms of the protein that can potentially enter the nucleus and activate transcription (1–2). Two cleavage sites were mapped at the aspartic acid residues at positions 154 and 398 (Asp154 and Asp398). However, no other caspase cleavage sites are underlined; the translation initiation codon is in bold); antisense, 5'-AGATATCATGAGCTCGGTTTCGCTG-3' (HindIII and NcoI restriction sites are underlined; the translation initiation codon is in bold); antisense primer was as for pHPI 1408. pHPI 1409, encoding a C-terminal-deleted form of NS5A lacking the last 94 amino acids, was constructed following amplification of the corresponding sequence from pHPI 611, HindIII digest, Klenow, and cloning into the XbaI-blunt-ended site of pCI. The primers used were: sense, 5'-CCAGCTCTGATACATGTTAGCATGGATCCGCTTGTG-3' (HindIII and NcoI restriction sites are underlined; the translation initiation codon is in bold); antisense, 5'-CTCGAGAAGCTTACGAGCATGCACGA-3' (XhoI and HindIII restriction sites are underlined; the complementary sequence of a stop codon is in bold); the antisense primer was as for pHPI 1408. pHPI 1407, encoding an N-terminal-deleted fragment of NS5A lacking the first 129 amino acids, was constructed following amplification of the corresponding sequence from pHPI 611, HindIII digest, Klenow, and cloning into the XbaI-blunt-ended site of pCI. The primers used were: sense, 5'-CCAGCTCTGATACATGTTAGCATGGATCCGCTTGTG-3' (HindIII and NcoI restriction sites are underlined; the translation initiation codon is in bold); antisense, 5'-ACCTCCGAGAAGCTTACGAGCATGCACGA-3' (XhoI and HindIII restriction sites are underlined; the complementary sequence of a stop codon is in bold). pHPI 1405, encoding an N-terminal NS5A fragment (1–233 amino acids), was constructed following ligation of the BamHI-PvuII blunt-ended fragment from pHPI 611 into the XbaI-blunt-ended site of pCI. pHPl 1570 encodes an N-terminal NS5A form (amino acids 1–248), that contains the mutations Asp154 → Glu154 and Arg217 → Gly217, and corresponds to the 31-kDa NS5A product. For the construction of pHPI 1570, firstly the two point mutations Asp154 → Glu154 and Arg217 → Gly217 were introduced sequentially in the NS5A coding sequence of pHPI 728, by using the primers described below, to yield plasmid pHPI 1439. Then, the nucleotide sequence encoding the mutated 31-kDa NS5A product was amplified by PCR, and inserted into the HindII site of pHUC19, yielding the plasmid pHPI 1569. The following primers were used: sense, 5'-AGATATCATGAGCTCGGTTTCGCTG-3' (EcoRV and SacI restriction sites are underlined; the translation initiation codon is in bold); antisense, 5'-CTCGAGAAGCTTACGAGCATGCACGA-3' (XhoI and HindIII restriction sites are underlined; the complementary sequence of a stop codon is in bold). The plasmid pHPI 1570 encodes an N-terminal NS5A fragment (amino acids 1–233), that contains the mutations Asp154 → Glu154 and Arg217 → Gly217, and corresponds to the 31-kDa NS5A product. For the construction of pHPI 1570, firstly the two point mutations Asp154 → Glu154 and Arg217 → Gly217 were introduced sequentially in the NS5A coding sequence of pHPI 728, by using the primers described below, to yield plasmid pHPI 1439. Then, the nucleotide sequence encoding the mutated 31-kDa NS5A product was amplified by PCR, and inserted into the HindII site of pHUC19, yielding the plasmid pHPI 1569. The following primers were used: sense, 5'-AGATATCATGAGCTCGGTTTCGCTG-3' (EcoRV and SacI restriction sites are underlined; the translation initiation codon is in bold); antisense, 5'-CTCGAGAAGCTTACGAGCATGCACGA-3' (XhoI and HindIII restriction sites are underlined; the complementary sequence of a stop codon is in bold). The plasmid pHPI 1570 encodes an N-terminal NS5A fragment (amino acids 1–233), that contains the mutations Asp154 → Glu154 and Arg217 → Gly217, and corresponds to the 31-kDa NS5A product. For the construction of pHPI 1316, the HindIII fragment

MATERIALS AND METHODS

Plasmids—All the plasmids were constructed using standard technology, and each time the sequence of the amplified NS5A fragment was verified by sequencing analysis (MWG-Biotech Co.). pHPI 728, expressing the full-length NS5A protein from HCV 1a strain H77, and pHPl 1403, expressing the N-terminal His₅-tagged full-length NS5A protein (NS5A/His), under control of the human cytomegalovirus (HCMV) immediate-early promoter, have been described before (65). pHPl 1408, carrying a small N-terminal-deleted form of NS5A, lacking the first 32 amino acids, was constructed following amplification of the corresponding sequence from pHPI 691 (65), HindIII digest, Klenow, and cloning into the XbaI-blunt-ended site of pCI. The primers used were: sense, 5'-CCAGCTCTGATACATGTTAGCATGGATCCGCTTGTG-3' (HindIII and NcoI restriction sites are underlined; the translation initiation codon is in bold); antisense, 5'-CTCGAGAAGCTTACGAGCATGCACGA-3' (XhoI and HindIII restriction sites are underlined; the complementary sequence of a stop codon is in bold); the antisense primer was as for pHPl 1408. pHPl 1409, encoding an N-terminal-deleted fragment of NS5A lacking the first 94 amino acids, was constructed following amplification of the corresponding sequence from pHPI 611 (66), HindIII digest, Klenow, and cloning into the XbaI-blunt-ended site of pCI. The primers used were: sense, 5'-CCAGCTCTGATACATGTTAGCATGGATCCGCTTGTG-3' (HindIII and NcoI restriction sites are underlined; the translation initiation codon is in bold); antisense, 5'-ACCTCCGAGAAGCTTACGAGCATGCACGA-3' (XhoI and HindIII restriction sites are underlined; the complementary sequence of a stop codon is in bold). pHPl 1405, encoding an N-terminal NS5A fragment (1–233 amino acids), was constructed following ligation of the BamHI-PvuII blunt-ended fragment from pHPI 611 into the XbaI-blunt-ended site of pCI. pHPI 1570 encodes an N-terminal NS5A form (amino acids 1–248), that contains the mutations Asp154 → Glu154 and Arg217 → Gly217, and corresponds to the 31-kDa NS5A product. For the construction of pHPI 1570, firstly the two point mutations Asp154 → Glu154 and Arg217 → Gly217 were introduced sequentially in the NS5A coding sequence of pHPl 728, by using the primers described below, to yield plasmid pHPl 1439. Then, the nucleotide sequence encoding the mutated 31-kDa NS5A product was amplified by PCR, and inserted into the HindII site of pHUC19, yielding the plasmid pHPI 1569. The following primers were used: sense, 5'-AGATATCATGAGCTCGGTTTCGCTG-3' (EcoRV and SacI restriction sites are underlined; the translation initiation codon is in bold); antisense, 5'-CTCGAGAAGCTTACGAGCATGCACGA-3' (XhoI and HindIII restriction sites are underlined; the complementary sequence of a stop codon is in bold). The plasmid pHPI 1570 encodes an N-terminal NS5A fragment (amino acids 1–233), that contains the mutations Asp154 → Glu154 and Arg217 → Gly217, and corresponds to the 31-kDa NS5A product. For the construction of pHPI 1316, the HindIII fragment

2 A. L. Epstein and V. Revol-Guyot, unpublished data.
from pHPI 691 (65), encoding the full-length NS5A, was blunt-ended and ligated into the blunt-ended XbaI site of the pA-EUA2 vector.

Finally, plasmid pHPI 1602 encodes the NS3 → NS5B polypeptide under the control of the HCMV promoter. The construction of this plasmid was as follows: (a) The Stul-HindIII NS3 → NS5B fragment (lacking the first 35 nt from NS3 and the last 1502 nt from NS5B) from pFL90 was cloned into the Smal-HindIII site of pUC19, giving rise to pHPl 1573. (b) For the reconstruction of the NS3 sequences, the N-terminal part of NS3 (nt 1–610) encoding the first 204 amino acids was amplified by PCR, using the following primers: sense, 5′-GGACATG-CTGCTAGCTAAAGGAGGCCCCATACAGGGCTACG-3′ (SpHl and XbaI restriction sites are underlined; the translation initiation codon is in bold); antisense, 5′-CCGGTGGGACATGCAAGGGCC-3′, and ligated into the HindIII site of pUC19, giving rise to pHPl 1574. (c) Next, the SpHl fragment containing the N-terminal part of NS3 (nt 1–610) from pHPl 1574 was inserted into the SpHl site of pHPl 1573, giving rise to pHPl 1669. (d) For the reconstruction of NS5B sequences, the C-terminal part of NS5B (nt273–1775) encoding the last 500 amino acids was amplified by PCR, using the following primers: sense, 5′-GCTATCCGTAGGAGAGC-GTTGCAGCTGGGC-3′ (nt 263–1775) and antisense, 5′-GGCCAGAGTTCCCTGTTGGGAGGAGTTAG-3′ ( HindIII and XbaI restriction sites are underlined; the complementary sequence of a stop codon is in bold); and ligated into the HindIII site of pHPl 1668, giving rise to pHPl 1666. (e) Following this, the HindIII fragment of this NS5B (nt 263–1775) from pHPl 1668 was inserted into the HindIII site of pHPl 1669, giving rise to pHPl 1670. (f) Finally, for expression in mammalian cells, the XbaI-blunt-ended fragment of the NS3 → NS5B coding sequence from pHPl 1670 was inserted into the XbaI-blunt-ended site of pA-EUA2, generating the pHPl 1674. All the chemicals were used within the indicated time.

Site-directed Mutagenesis—Site-directed mutagenesis was performed by using the QuikChange™ Site-directed Mutagenesis kit (Stratagene), as specified by the supplier. All the sequences were then verified by sequencing analysis (MWG-Biotech Co.). Using the pHPl 728 (see above), the Asp154 (GAC) was converted to Glu154 (GAA), giving rise to pHPl 1426. The primers used were: sense, 5′-CTAGTTCACTAGCTACCATGGCCCTCCGGCTCGTGCC (XbaI and Ncol restriction sites are underlined; the translation initiation codon is in bold); antisense, 5′-CTAGTCAGACTAGCTACATGCC (the mutated codon and its complementary sequence are in bold), or sense, 5′-CTAGTCAGACTAGCTACATGCC (the mutated codon and its complementary sequence are in bold), or sense, 5′-TGGAGGAGTGGCCAGACACGTG-3′ (XbaI restriction site is underlined; the complementary sequence of a stop codon is in bold).

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Immunoblot Analysis, Antibodies, and Immunofluorescence Analysis—Cell monolayers were harvested at the indicated times p.t., rinsed with ice-cold PBS-A and lysed (10 min on ice) in triple detergent buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% SDS, 100 μg/ml of phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.5% sodium deoxycholate), in the presence of protease inhibitor mixture (Sigma), as specified by the manufacturer. Approximately 30–40 μg of total protein were analyzed each time, unless indicated otherwise. Following this, SDS-polyacrylamide gel electrophoresis loading buffer was added to each sample, the samples were boiled for 3 min, separated on a dena-
Caspase-mediated Cleavage of the HCV 1a-NS5A

**FIGURE 1.**

**A.**

![Caspase-mediated Cleavage of the HCV 1a-NS5A](image)

**B.**

![Caspase-mediated Cleavage of the HCV 1a-NS5A](image)

**C.**

![Caspase-mediated Cleavage of the HCV 1a-NS5A](image)

**RESULTS**

Caspase-mediated Cleavage of NS5A—Earlier studies from this laboratory have shown that NS5A from genotype 1a was cleaved in two C-terminally truncated fragments (40 and 24 kDa), when expressed in transiently transfected Vero cells, two additional bands of 31 and 17 kDa in size were detected. Interestingly, when NS5A was expressed in transiently transfected Vero cells, two additional bands of 31 and 17 kDa in size were detected. Interestingly, the accumulation of the latter fragments was unaffected by the use of calpain proteases were responsible for the generation of both fragments, whereas the accumulation of the 24-kDa fragment was also dependent on caspase activity (65). However, when NS5A was expressed in transiently transfected Vero cells, two additional bands of 31 and 17 kDa in size were detected. Interestingly, the accumulation of the latter fragments was unaffected by the use of calpain inhibitors, whereas the use of the pannacase inhibitor Z-VAD-FMK completely blocked their production (65), suggesting that both the 31- and 17-kDa proteins may represent caspase-dependent NS5A cleavage.

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TABLE 1
Plasmids used in this study

| Plasmid   | Vector       | Description of insert                                  |
|-----------|--------------|-------------------------------------------------------|
| pHPI 691  | pGEM-3zf (+) | Entire NS5A coding sequence (nt 6258–7601) from HCV 1a (65) |
| pHPI 728  | pCI          | PCR product of N-terminal-deleted (-162 aa) NS5A from pHPI 691 (66) |
| pHPI 1406 | pCI          | HindIII fragment encoding the entire NS5A from pHPI 691 (65) |
| pHPI 1403 | pA-EUA2      | PCR product of N-terminal-deleted (-32 aa) NS5A from pHPI 691 (65) |
| pHPI 1316 | pA-EUA2      | PCR product of N-terminal-deleted (-129 aa) NS5A from pHPI 691 (66) |
| pHPI 1408 | pCI          | PCR product of N-terminal-deleted (-235 aa) NS5A from pHPI 691 (66) |
| pHPI 1411 | pCI          | BamHI-PvuII fragment encoding the first 233 aa of NS5A from pHPI 691 (66) |
| pHPI 1407 | pCI          | PCR product of C-terminal-deleted (-94 aa) NS5A from pHPI 691 (65) |
| pHPI 1409 | pCI          | PCR product of N-terminal-deleted (-162 aa) NS5A from pHPI 691 (66) |
| pHPI 1505 | pUC19        | PCR product of the full-length NS5A protein (data not shown). Collectively, these results indicate that Asp154 Glu248 or of the NS5A/Asp248 Glu154 results in the loss of glutamic acid residues (Table 1). The proteolytic processing of the NS5A protein was analyzed in Vero, HeLa, and WR-L68 cells transfected separately with plasmid pHPI 728 expressing the wild-type NS5A protein (Fig. 2, A and B, lane 2), or plasmids expressing the mutated forms of NS5A. These included plasmids pHPI 1426 expressing the NS5A/Asp154 → Glu154 form (Fig. 2A, lane 3), pHPI 1564 expressing the NS5A/Asp251 → Glu251 form (Fig. 2B, lane 3), and pHPI 1567 expressing the NS5A/Asp165 → Glu165 form (Fig. 2C, lane 3). Cells were harvested at 48 h p.t., and the proteins were visualized by Western blot analysis with the NS5A polyclonal antibody. As shown in Fig. 2A, the mutation Asp154 → Glu154 results in the loss of the 17-kDa protein (band d) in all cell lines tested. Furthermore, expression of the mutated NS5A/Asp248 → Glu248 or of the NS5A/Asp251 → Glu251 proteins result in the loss of the 31-kDa product (band d) (Fig. 2B, lanes 3 or 4, respectively). On the other hand, the mutation Asp251 → Glu251 has no effect on the cleavage pattern of the NS5A protein (data not shown). Collectively, these results indicate that Asp154 as well as the 248DXXD251 sequence belong to bona fide caspase recog-
nition sites, which are responsible for the production of the 17-kDa and 31-kDa products, respectively. Notably, Asp154 has been previously identified as a caspase recognition site for the NS5A of HCV-1b (2), whereas the 248DXXD251 is a novel caspase recognition site that represents a consensus sequence among the majority of HCV isolates. On the other hand, the Asp205 does not appear to belong to a caspase recognition motif, as its conversion to Glu205 had no effect on the NS5A proteolytic processing.

For the production of the 24-kDa NS5A fragment, it is predicted that a putative cleavage within the amino acid stretch between residues 200 and 235 of the NS5A sequence could produce an N-terminal product of ~24 kDa in size. However, provided that apart from the Asp205, no other predicted caspase recognition motifs were identified within this sequence, and that the generation of the 24-kDa fragment is sensitive to both calpain and caspase inhibitors, we assumed that the 24-kDa fragment is the result of calpain-mediated cleavage, whereas caspasas may modulate this cleavage (67–69). As calpain recognition sites are not well defined, our first attempt to identify the cleavage site for the 24-kDa fragment was to examine the cleavage pattern of a number of NS5A mutated forms carrying multiple changes within the corresponding region of NS5A protein. The first construct contained three mutations at positions 224, 225, and 227 (Pro224 → Ser224, Ser225 → Pro225, and Ala227 → Asp227), the second carried four mutations, targeting simultaneously the above three amino acids as well as the residue at position 205 (Asp205 → Gly205), the third contained five mutations targeting simultaneously the above four amino acids as well as the residue at position 232 (Ser232 → Ile232), and the fourth NS5A mutant combined the five previously described changes with an alteration at position 217 (Arg217 → Gly217) (Table 1). Whereas none of the first three NS5A mutants had an effect on NS5A cleavage, the latter suppressed the production of the 24-kDa product, suggesting that Arg217 may be a critical amino acid in the generation of this product (data not shown).

To test this possibility further, two additional mutated NS5A forms were constructed, with a conversion of arginine at position 217 to either glycine (Arg217 → Gly217) or to lysine (Arg217 → Lys217). Vero, HeLa, or WRL68 cells were transfected separately either with the plasmid pHPI 1438 expressing the mutated NS5A/Arg217 → Gly217 form (lane 3), with pHPI

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FIGURE 2. Mapping the caspase recognition sites in the NS5A protein. A, Vero, HeLa or WRL 68 cells, seeded in 12-well plates, were transfected either with the empty vector pCI (lane 1), with pHPI 728 (lane 2), with pHPI 1565 (Asp248 → Glu248) (lane 3), or with pHPI 1423 (Asp251 → Glu251) (lane 4). Cells were harvested at 48 h.p.t., and proteins were visualized by Western blot analysis with the NS5A polyclonal antibody. An overexposure of the bottom of the gel is shown for WRL 68. B, Vero, HeLa, or WRL 68 cells, seeded in 12-well plates, were transfected either with the empty vector pCI (lane 1), with pHPI 1565 (Asp248 → Glu248) (lane 2), with pHPI 1565 (Asp248 → Glu248) (lane 3), or with pHPI 1423 (Asp251 → Glu251) (lane 4). Cells were harvested at 48 h.p.t., and proteins were visualized by Western blot analysis with the NS5A polyclonal antibody, as above. Arrow and a–d denote the full-length NS5A and its cleavage products, respectively. Molecular mass markers are shown on the right.

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FIGURE 3. Mapping the cleavage site for the 24-kDa NS5A product. A–C, Vero, HeLa, or WRL 68 cells, seeded in 12-well plates, were transfected either with the empty vector pCI (lane 1), with pHPI 728 (lane 2), with pHPI 1438 (lane 3), or with pHPI 1564 (lane 4). Cells were harvested at 48 h p.t., and proteins were visualized by Western blot analysis with the NS5A polyclonal antibody, as above. Arrow and a–d denote the full-length NS5A protein and its cleavage products. Molecular mass markers are shown on the right.

Caspase-mediated Cleavage of NS5A Does Not Require an Intact NS5A Protein—As previous studies have shown that the NS5A protein is anchored on the ER membrane through an N-terminal amphipathic α-helix (40), we sought to examine whether caspase activation was the result of ER stress because of overexpression of NS5A in transfected cells. To assess this, we examined the cleavage pattern of several NS5A deletion mutants, which lack the N-terminal part of the protein that contains the ER anchoring signal (Fig. 4A). For this purpose, Vero cells were separately transfected with plasmids expressing the NS5A lacking either the first 129 (pHPI 1411), 162 (pHPI 1406), or 235 (pHPI 1407) amino acid residues (Fig. 2B), or with a plasmid expressing an NS5A deletion lacking only the first 32 amino acids (pHPI 1408) (Fig. 4C). Thirty-six hours p.t., cells were either treated with the Z-VAD-FMK inhibitor (Fig. 4B, lanes 3, 5, 7, and 9) or remained untreated (Fig. 4B, lanes 1, 2, 4, 6, and 8), and cell lysates were analyzed by Western blot, as above. As shown in Fig. 4, the production of all N-terminal-truncated forms of NS5A was followed by the appearance of shorter forms of the protein. Interestingly, as shown in B, the accumulation of a 22-kDa fragment (marked by arrows) was inhibited by the presence of the pan-caspase inhibitor (lanes 5, 7, and 9), suggesting the involvement of a caspase-mediated cleavage. In contrast, the accumulation of a 33-kDa fragment (marked by stars) remained unaffected in the presence of Z-VAD-FMK, but was affected by calpain inhibitors (data not shown). We assume that both the 22- and 33-kDa cleavage products may represent C-terminal parts of the protein generated by caspase-mediated cleavage at position Asp251, or by calpain-mediated cleavage near Arg217, respectively, which are produced by an as yet unknown mechanism in the selected N-terminal-truncated forms of NS5A. Notably, the NS5A form lacking only the first 32 amino acids was also cleaved in transfected cells, generating the expected N-terminal forms of NS5A (shown by arrow), shorter by 33 amino acids (Fig. 4C, lane 3). To confirm the intracellular localization of the N-terminal forms of NS5A, we performed immunofluorescence studies (Fig. 4E). As expected all N-terminally deleted forms of NS5A exhibit a diffuse cytoplasmic-nuclear staining pattern (Fig. 4E, panels c, d, e, and f), compared with the full-length NS5A (panel b). Collectively, these data suggest that the induction of caspase cleavage of the NS5A protein in transfected cells is independent of the accumulation of the protein on ER membranes.

Finally, we investigated whether the NS5A protein can be proteolytically processed in the absence of its C-terminal part. For this purpose, two C-terminally truncated forms of NS5A were constructed, encoding either the first 233 (pHPI 1405) or the first 354 amino acids of the protein (pHPI 1409), respectively (Fig. 4A), and their expression was analyzed in transiently transfected Vero cells. As shown in Fig. 4D, both the C-terminal-truncated NS5A forms are proteolytically processed to produce the expected N-terminal fragments, namely the 17-, 24-, 31-, and 40-kDa fragments for pHPI 1409 (lane 3), and the two shorter fragments for pHPI 1405 (lane 4). In contrast, when amino acid substitutions Asp154 → Glu154 and Arg217 → Gly217 were introduced into a plasmid that encodes for the 31-kDa form of NS5A, the production of the 17- and 24-kDa fragments was abolished (lane 5). Collectively, these data demonstrate that neither the N- nor the C-terminal part of the NS5A protein are required for its proteolytic processing by caspases and calpains.

Identification of the Caspases Involved in NS5A Cleavage—To determine which caspase(s) are responsible for the NS5A cleavage, firstly we examined the effect of various specific caspase inhibitors on the accumulation of the 17- and 31-kDa NS5A products (Fig. 5). For this purpose, Vero and WRL 68 cells (data not shown) transiently expressing NS5A were treated separately for 12 h with the pancaspase inhibitor (Z-VAD-FMK; 50 μM) (A, lane 4; B and C, lane 3) or with 50 μM of the most potent inhibitors for caspase 3 (DEVD-CHO) (A, lane 3), caspase 9 (Ac-LEHD-CHO) (A, lane 5) caspase 8 (Z-IETD-FMK) (B, lane 2), caspase 2 (Ac-VDVAD-CHO) (C, lane 4), caspase 1 (YVAD-CHO) (C, lane 5), caspase 6 (Ac-DEVD-CHO) (C, lane 6), or with the calpain inhibitor (Gly217 mutant results in Gly217) and the two shorter fragments for pHPI 1405 (lane 4). In contrast, when amino acid substitutions Asp154 → Glu154 and Arg217 → Gly217 were introduced into a plasmid that encodes for the 31-kDa form of NS5A, the production of the 17- and 24-kDa fragments was abolished (lane 5). Collectively, these data demonstrate that neither the N- nor the C-terminal part of the NS5A protein are required for its proteolytic processing by caspases and calpains.
FIGURE 4. Proteolytic processing of the N- and C-terminal-deleted forms of NS5A. A, schematic representation of the full-length NS5A protein, depicting the location of some of the major structural components, including the amphipathic α-helix, the hyperphosphorylation sites (P), and the NLS. Asp154 and Asp248/Asp251 represent the two caspase recognition motifs identified in this study. Arg217 represents a calpain-dependent motif identified also in this study. Schematic representation of the N- and C-terminal NS5A-deleted forms expressed from the corresponding plasmids. B, Vero cells, seeded in 12-well plates, were transfected with pHPI 728 (lanes 2 and 3), with pHPI 1411 (lanes 4 and 5), with pHPI 1406 (lanes 6 and 7), with pHPI 1407 (lanes 8 and 9), or with the empty vector pCI (lane 1). 36 h.p.t., cells were either treated for 12 h with the pancaspase inhibitor Z-VAD-FMK (50 μM) (lanes 3, 5, 7, and 9), or left untreated (lanes 1, 2, 4, 6, and 8). Cells were collected at 48 h.p.t., lysed, and proteins were separated on a denaturing 12% polyacrylamide gel and visualized by Western blot analysis with the NS5A polyclonal antibody. An overexposure of parts of the gel, indicated by brackets is illustrated below. Stars denote the calpain-dependent NS5A cleavage fragments. Arrows denote the caspase-dependent NS5A cleavage fragments. Molecular mass markers are shown on the right.

C, Vero cells, seeded in 12-well plates, were either mock-transfected (lane 1), or transfected with pHPI 728 (lane 2), or with pHPI 1408 (lane 3). Cells were harvested at 48 h.p.t., proteins were visualized by Western blot analysis with the NS5A polyclonal antibody, as before. Arrows denote the cleavage products of the full-length NS5A protein and their putative corresponding fragments, as predicted after the removal of the first 32 amino acids of the protein. Overexposure of the bottom of the gel, which corresponds to a molecular mass lower than 17 kDa, is also visualized.

D, Vero cells, seeded in 12-well plates, were either transfected with pCI (lane 1), with pHPI 728 (lane 2), with pHPI 1409 (lane 3), with pHPI 1405 (lane 4), or with pHPI 1570 (lane 5). Cells were harvested at 48 h.p.t., lysed, and proteins were separated on a denaturing 12% polyacrylamide gel and visualized by Western blot analysis with the NS5A polyclonal antibody. Arrow and a–d denote the full-length NS5A and its cleavage products. Molecular mass markers are shown on the right. E, Vero cells, seeded on 10-mm cover glasses, were either mock-transfected (panel a), or transfected with pHPI 728 (panel b), with pHPI 1411 (panel c), with pHPI 1406 (panel d), with pHPI 1407 (panel e), or with pHPI 1408 (panel f). At 48 h.p.t., cells were fixed with 3.7% paraformaldehyde and immunofluorescence analysis was performed by using the NS5A polyclonal antibody.
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FIGURE 5. Inhibition of the NS5A cleavage by various caspase inhibitors and in vitro cleavage of the NS5A protein by caspases. Vero cells (A–D), seeded in 12-well plates, were transfected either with pHPl 728 (A, lanes 2–5; B, lanes 1–3; C, lanes 2–5; D, lanes 2–4), or with the empty vector pC1 (A, lane 1; C, lane 1; D, lane 1). 36 h p.t., cells were separately treated for 12 h with 50 μM Z-Val-DEVD-FMK (pan-caspase inhibitor) (A, lane 4; B and C, lane 3), with DEVD-CHO (caspase 3 inhibitor) (A, lane 3), with Ac-LEHD-CHO (caspase 9 inhibitor) (A, lane 5), with Z-Val-DEVD-FMK (caspase 8 inhibitor) (B, lane 2), with Ac-VDVAD-CHO (caspase 2 inhibitor) (C, lane 4), with YVAD-CHO (caspase 1 inhibitor) (C, lane 5), with Ac-VEID-CHO (caspase 6 inhibitor) (D, lane 3), with Z-AEVDE-FMK (caspase 10 inhibitor) (D, lane 4), or remained untreated (A, lanes 1 and 2; B, lane 1; C, lanes 1 and 2, D, lanes 1 and 2). Cells were collected at 48 h p.t., lysed, and proteins were separated on a denaturing 12% polyacrylamide gel and visualized by Western blot analysis with the NS5A polyclonal antibody. Overexpression of the bottom part of the gels in C and D for better visualization of the 17-kDa NS5A fragment is also illustrated. Arrow and a–d denote the full-length NS5A and its cleavage products. Molecular mass markers are shown on the right. In vitro translation (IVT) of the wt NS5A and its mutated forms (E), by using the TNT transcription-translation kit (lanes 1–4). Equal amounts from the in vitro translation reactions of the wt NS5A or its mutants were incubated either with 250 units of caspase 2 (lanes 5–8) or with 250 units of caspase 6 (lanes 9–12), for 6 h at 37 °C, as described under “Materials and Methods.” Following, proteins were analyzed on a denaturing 12% polyacrylamide gel and visualized by autoradiography. Molecular mass markers are shown on the right. Arrow denotes the full-length NS5A protein. Stars denote the 17-kDa NS5A cleavage products. Arrowheads denote the cleaved NS5A protein.

To validate the involvement of caspases 2 and 6 in the cleavage of the NS5A protein, we performed an in vitro cleavage assay. As expected, the use of Z-Val-DEVD-FMK blocked the production of both the 31- and the 17-kDa products, whereas it partially reduced the production of the 24-kDa protein. In contrast, treatment with inhibitors for caspases 1, 3, 8, 9, and 10 had no apparent effect on the proteolytic processing of the NS5A protein. Interestingly, however, the use of the most potent inhibitor for caspase 2 blocked the production of the 31-kDa protein (band b), whereas the production of the 17-kDa protein (band d) was slightly decreased. Conversely, the use of the most potent inhibitor for caspase 6 severely inhibited the production of the 17-kDa protein (band d) with no apparent effect on the 31-kDa protein (band b). Notably, the production of the 24-kDa protein (band c) remains completely unaffected in the presence of the caspase 2 or the caspase 6 inhibitors. These data suggest that NS5A is targeted by at least two different caspasases in transfected cells in the absence of exogenous apoptotic stimuli.

To verify that the NS5A cleavage fragments are cleaved in vivo, we performed an in vitro cleavage assay. As expected, the 35S-labeled NS5A protein, which was produced from rabbit reticulocyte lysates was used as a substrate for the recombinant caspases 2 and 6, respectively. Mutated forms of the NS5A protein where the previously described putative cleavage sites for the caspases 2 and 6 have been modified, serve as negative controls. As it is illustrated in Fig. 5E, caspase 6 efficiently cleaves the wt NS5A protein in vitro, to produce the 17-kDa fragment. As expected, the production of the 17-kDa fragment by caspase 6 is abolished when the Asp154 is converted to Glu154 (lane 12) and remains unaffected when the Asp248 or the Asp251 are converted to Glu248 or Glu251, respectively (lanes 10 and 11). On the other hand, treatment by caspase 2 failed to produce the 31-kDa fragment. Instead a smaller by 2 kDa NS5A form is efficiently produced suggesting cleavage close to the N or C terminus of the protein. Notably, there are several putative caspase recognition sites at the C-terminal part of NS5A that could be potentially used by caspase 2 under our experimental conditions. Collectively, these data suggest that caspase 6 is directly involved in the production of the NS5A 17-kDa fragment. However, the possible involvement of caspase 2 on the production of the 31-kDa fragment needs further investigation.

Finally, as previous studies have shown that NS5A protein does not induce apoptosis in transfected cells, it was of interest to examine the apoptotic conditions in our experimental settings (52–54, 56–57, 71). For this purpose, WRL 68 or Vero cells (data not shown) transiently expressing NS5A were assayed for apoptosis by a trypan blue exclusion assay. To monitor the cells that express NS5A, we modified our expression vector to contain, in addition to the full-length NS5A sequence, the GFP gene under a different promoter (pHPl 1316). Cells were transfected with the above plasmid, and at 48 h p.t., they were treated with trypan blue. Subsequently, microscopy analysis was performed, and the percentage of the green cells that had been stained with trypan blue in comparison to the total number of green cells measured in the analyzed fields was estimated. As a negative control, cells transfected with the plasmid vector that expressed only GFP were used. As a positive control cells transfected with the same plasmid vector were treated with TNFα (0.01 ng/ml) and CHX (50 μg/ml) for 5 h to induce apoptosis. As illustrated in Fig. 6A, only 6.7% of the cells that expressed the NS5A protein were stained with trypan blue. This result was comparable to that of the negative control, whereas the percentage of green cells stained with trypan blue after treatment with TNFα and CHX was ~72.4%. To verify these results, the condensation of chromatin in the nucleus of NS5A-expressing WRL68 cells was also investigated by Hoechst 333258 staining, under the above experimental conditions. Following the analysis of several fields, representative data shown in Fig. 6B indicate no chromatin condensation in NS5A-expressing cells (panel c) or empty vector-
transfected cells (panel b) compare with the TNFα/CHX-treated cells (panel d). Finally, the caspase 3 activity in NSSA-transfected WRL68 cells was also tested by investigating the cleavage of one of its substrate, PARP. As it is illustrated in Fig. 6C PARP cleavage is detected only in TNFα/CHX-treated cells and not in mock-, NSSA-, or empty vector-transfected cells. Collectively, these data demonstrate that in agreement with previous studies, the transient expression of NSSA protein does not lead to apoptosis.

NSSA from Genotype 1a Is Proteolytically Processed in the Presence of the HCV Nonstructural Proteins—To investigate the effect of the other HCV proteins on the caspase-mediated cleavage of NSSA, plasmid pHPl 1602, expressing the NS3-NS4A-NS4B-NS5A-NS5B nonstructural proteins of HCV 1a under the HCMV promoter, was constructed. HeLa cells were previously transfected separately with pHPI 1316, which encodes the full-length NS5A (lane 2), or with pHPI 1602 (lane 3), and at 48 h.p.t. proteins were analyzed by immunoblotting, as before. As illustrated in Fig. 7A (lane 3) NS5A is proteolytically processed in the presence of the other viral proteins, generating the 31-, 24-, and 17-kDa cleavage products, suggesting that caspase-mediated processing of NS5A is compatible to the expression of most HCV nonstructural proteins. On the other hand, all attempts to detect NSSA cleavage when the con.1 replicon was used were unsuccessful, most likely because of the low level of NSSA protein expression (data not shown). However, as the con.1 replicon system is derived from the HCV-1b genotype, we also sought to examine the proteolytic cleavage of NSSA of HCV-1b under our experimental conditions. For this purpose Vero cells were either mock-transfected Fig. 7B (lane 1), transfected with pHPI 728, which expresses the full-length NS5A 1a (lane 2), or with pHPI 1663, which expresses the full-length NS5A 1b (lane 3), and analyzed by Western blotting, as before. As illustrated in Fig. 7B, the NSSA of HCV-1b is cleaved to produce a 17-kDa (band d), 24-kDa (band c), and 31-kDa (band b) fragment, implying cleavage at sites Asp154, Arg157, and Asp251, as for the NSSA of HCV-1a. Interestingly, however, the calpain-dependent N-terminal 40-kDa fragment (band a) is not produced from the NSSA of HCV-1b (65). Instead, a new product of 48 kDa is detected, as reported in previous studies (2). Thus, although our data support cleavage of NSSA in the presence of the viral nonstructural proteins constituting the replication complex, the relationship between NSSA cleavage and virus replication remains to be elucidated.

**DISCUSSION**

We report here that the NSSA protein from HCV genotype 1a is cleaved by caspases to produce shorter, N-terminal forms of the protein, in the absence of exogenous apoptotic stimuli. Two cleavage sites at the aspartic residues Asp154 and Asp251 were mapped. Cleavage at Asp154 has been previously recognized as one of the caspase cleavage sites for the NSSA protein of HCV genotype 1b (1–2) and results in the production of a 17-kDa N-terminal NSSA fragment. The sequence 248DXXD251 is a novel caspase recognition motif for NSSA that represents a consensus sequence among the majority of HCV genotypes and is responsible for the production of a 31-kDa N-terminal NSSA fragment. Interestingly, the use of caspase-specific inhibitors combined with an in vitro cleavage assay has implicated caspase 6 in the cleavage of NSSA at position Asp154. On the other hand, although the production of the 31-kDa fragment was blocked in transfected cells by the addition of a caspase 2-specific inhibitor, the purified caspase 2 failed to generate this fragment in vitro. Thus, the identity of the caspase responsible for cleavage at 248DXXD251 position remains ambiguous. It is possible that changes in the conformation of the in vitro synthesized NSSA substrate, or an indirect role of caspase 2 to be responsible for the failure to produce the 31 kDa in vitro. Alternatively, other caspases that could be blocked by the Ac-VDVAD-CHO inhibitor may be responsible for the production of the 31-kDa fragment. Nevertheless, our data show that at least two different caspases target NSSA, and it appears that there is no...
interplay between those cleavage events in our studies. Notably, the

\[\text{DXXD}^{251}\]

motif represents a recognition site for other caspases and thus may be recognized under conditions, which permit the activation of those caspases (72). Furthermore, we showed that the arginine residue at position 217 (Arg\(^{217}\)) is implicated in the production of the previously described 24-kDa N-terminal NS5A fragment, whose accumulation is affected by both calpain and caspase inhibitors. Because no known recognition motif for caspases is present in the respective region of NS5A, it could be predicted that Arg\(^{217}\) contributes to a calpain recognition motif. Interestingly, we also found that N-terminally truncated forms of the protein are still processed by caspases suggesting that caspase activation does not correlate with the accumulation of NS5A on the ER membranes. Finally, cleavage of the NS5A protein was also observed in the presence of most HCV nonstructural proteins (NS3-NS4A-NS4B-NS5A-NS5B) (Fig. 7A).

One of the most intriguing findings of our work was that only N-terminal fragments of the NS5A protein, which carry the ER associating signal, were detectable following caspase cleavage, whereas detection of the corresponding C-terminal fragments remained elusive. Consistent with these findings, nuclear localization of the full-length NS5A protein under conditions that allow caspase-mediated cleavage was not detected. Therefore, in sharp contrast to the current view, our data failed to provide evidence supporting a role for caspases in NS5A nuclear translocation following proteolysis as a mechanism of transcriptional activation (1–2, 42–43). Instead, it seems that the NS5A cleavage favors NS5A functions located in the N-terminal part of the protein and are mainly linked with properties of the cell membranes (Fig. 8) (65). Notably, several recent studies have shown that NS5A can activate transcription, while localized to the cytoplasm. Siddiqui and co-workers (62, 63) have shown that NS5A induces activation of STAT3 and NF-\(\kappa\)B via disruption of intracellular calcium levels. Similarly, Park et al. (56, 73) have shown that NS5A modulates the TRAF2-mediated JNK activity, thus affecting c-Jun-mediated transcription, whereas the NS5A-TRAF2 protein interaction was also shown to inhibit TNF-\(\alpha\)-induced NF-\(\kappa\)B activation. Furthermore, Qadri et al. (51) reported that NS5A interaction with p53 and TBP affects transcription, and Ghosh et al. (49) showed that NS5A through its direct interaction with the transcriptional factor SRCAP may exert its negative effect on the p21 promotor. Notwithstanding, Yeh et al. (74) who have recently characterized a mutant form of NS5A isolated from a patient with hepatocellular carcinoma, have shown that although the protein was localized both in the cytoplasm and the nucleus, its transactivation properties were unrelated to nuclear localization.

As NS5A is known to be the classical antiapoptotic protein of HCV virus (52–56, 61, 71), the biological relevance of our findings remains unclear. However, a growing number of studies reveal novel nonapoptotic activities of caspases, indicating that caspases are much more versatile enzymes than originally expected (7–10, 75–76). For example, caspase 3 and caspase 11 mediate IL-1 production (8, 77–78). Caspase 3 is involved in the terminal differentiation of erythrocytes, keratinocytes, monocytes, and epithelial, sperm, skeletal, muscle, osteoblast, and trophoblast cells as well as in B cell proliferation (8–9, 79–84). Caspase 8 mediates T cell proliferation, placental, and trophoblast differentiation as well as cell cycle control (7, 9, 85–86). Caspase 12 attenuates inflammatory and innate immune responses (87). Caspase 14 mediates terminal differentiation of keratinocytes (85, 88). Finally, caspase 2 has been shown to participate in the activation of the DNA repair machinery when it is activated as a part of a multiprotein complex known as the PIDDsome complex (89). Additionally, proteins from different families seem to serve as substrates for caspase 2, such as golgin-160, PKC\(\delta\), and all-spectrin, implying that caspase 2 can act both as a signaling and an executioner caspase (90–92). Furthermore, many examples of molecules cleaved by caspases in nonapoptotic cells have been described. Recently, it was shown that the \(\beta\)-subunit in the IKK\(\beta\) complex can be inactivated by caspase 3 (93), and the p50 and p65 subunits of NF-\(\kappa\)B are substrates for caspase 3, resulting in the loss of their transcriptional activity (94–95). Molecules that are implicated in cell proliferation pathways, such as MEK, STAT1, CREB, PKC, and p51 (96–100) or molecules involved in cell cycle regulation, such as the cyclin inhibitors p21\(^{\text{Cip}}\)/\(\text{Wat}\) and p27\(^{\text{Kip}}\), as well as the protein kinase Wee1, are also caspase substrates. Notably, cleavage of Wee1 by caspases mediates progression through the cell cycle (101). Thus, it appears that caspases have a dual function in life and death (8), and the selectivity of substrate cleavage in nonapoptotic cells is likely to be achieved either by a compartmentalization of caspases, activation of antiapoptotic factors or through limited activity of caspases (102).

The impact of caspase-mediated cleavage of NS5A on the virus life cycle is presently unknown. It can be argued that NS5A is cleaved by caspases because it happens to contain caspase cleavage sites in its sequence. On the other hand, however, it is now well recognized that caspase-mediated cleavage of many viral proteins is a direct requirement for propagation of those viruses (10). Specifically, caspase 2 has been implicated in the cleavage of FCV capsid (14), whereas caspase 6 was found to be involved in the processing of human astrovirus H\(\alpha\)STV capsid precursor (12), in TGEV.
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Transcriptional activation domain (42, 107)
PKR binding domain (44, 108)
Karyopherin β3 interaction (109)
TRAF2 interaction (56)
NS5B binding (37)
HSP70 interaction (110)
Apaf1 interaction (60)
2-5 AS interaction (111)
Grb2 interaction (112-113)
IRES suppression (34)
La interaction (114)
Amphiphysin II interaction (115)
p53 interaction (50-51, 55)
H5TA5F32 (55)
Homodomain protein PTX1 (116)
Phosphoinositide-3-kinase p85 subunit (57, 117)
Scc-family kinases, Fyn, Lck and Hck (112)
Scc-family kinase Lyn (112)

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