Kobe J. Med. Sci., Vol. 50, No. 5, pp. 153-166, 2004

# Cleavage of the Hepatitis C Virus NS5A Protein by Caspase-3 in the Interferon Sensitivity-Determining Region in a Sequence-Dependent Manner

RACHMAT HIDAJAT, MOTOKO NAGANO-FUJII, LIN DENG, and HAK HOTTA*

**Corresponding author. Mailing address: Division of Microbiology, Department of Genome Sciences, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan.*

Received 15 February 2005/ Accepted 22 February 2005

**Keywords***:* Hepatitis C virus, NS5A, Caspase, Cleavage

**Abbreviations:** HCV, hepatitis C virus; amino acid(s), aa; FCS, fetal calf serum; cycloheximide, CHX; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IFN, interferon; ISDR, IFN sensitivity determining region

**Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) has versatile functions and has been implicated in viral pathogenesis, including interferon (IFN) resistance and hepatocarcinogenesis. It has been reported that NS5A is cleaved into a few fragments by a caspase(s) or caspase-like enzyme(s) under certain conditions. Two cleavage sites have been mapped to the Asp residues at positions 154 and 398 ($D^{154}$ and $D^{398}$). However, other possible cleavage sites were not determined yet so far. In this study, we demonstrated caspase-3-mediated NS5A cleavage upon apoptotic stimuli and identified a new site as the third cleavage target. This site was mapped to $D^{251}$, which lies within IFN sensitivity-determining region (ISDR). Although $D^{251}$ was conserved among all HCV subtype 1b (HCV-1b) strains tested, the consensus caspase-3 recognition sequence ($D^{248}$-X-X-$D^{251}$) was not conserved due to the sequence variation at position 248 in ISDR. Furthermore, $A^{252}$ was found to be necessary for efficient cleavage of NS5A. The virological significance of the HCV strain-dependent NS5A cleavage at this site awaits further investigation.**

## INTRODUCTION

Hepatitis C virus (HCV), a member of the Flaviviridae family, is known to be a causative agent of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (25,27,28). The genome of the virus is a single-stranded, positive-sense RNA of approximately 9.6 kb in length. The viral genome shows a considerable degree of sequence variation, based on which HCV is currently classified into at least 6 clades consisting of more than 60 genotypes (subtypes) (4,22,24). Geographic distribution and clinico-pathological features including interferon (IFN) responsiveness appear to vary with different HCV genotypes (1,4,22,29,30). The HCV genome consists of 5'- and 3'- untranslated regions and a large open reading frame encoding a polyprotein of about 3,000 amino acids (aa) that undergoes proteolytic processing

Phone: (81) 78-382-5500 Fax: (81)78-382-5519 E-mail: hotta@kobe-u.ac.jp 

by both signal peptidase of the host cell and two virally-encoded proteinases to generate at least 10 viral proteins; core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (21).

HCV NS5A is a membrane-associated phosphoprotein of 447 residues, and is found in a basally phosphorylated form of 56 kDa and a hyperphosphorylated form of 58 kDa. Phosphorylation occurs mostly at serine residues and a minor fraction of threonine residues (16). NS5A is reported to be involved in HCV resistance to IFN-α, the main treatment used for HCV infection at present. Enomoto *et al.* (5,6) reported a strong correlation between the number of mutations in a region spanning from aa 237 to 276 of NS5A and IFN treatment efficacy in patients infected with HCV genotype 1b (HCV-1b) and therefore, this region is called the IFN sensitivity-determining region (ISDR) (5,6). Some studies in Japan observed that most patients infected with HCV strains possessing 4 aa mutations or more in the ISDR experienced sustained responses to IFN, even after the termination of the therapy. In addition to that observation, NS5A was also shown, through the ISDR and its adjacent region, to interact with and interfere the activity of double-stranded RNA-dependent protein kinase (PKR), one of the key molecules for IFN antiviral activity (8). However, the ISDR function remains controversial. Some investigators reported data similar to those of the Japanese studies (12,34), while others could not find a significant correlation between the number of mutations in ISDR and IFN efficacy (9,23). Further investigations are still needed to refine the function of ISDR mutations on clinical efficacy of IFN treatment.

When expressed in mammalian cells, NS5A is localized in the perinuclear region of the cytoplasm. NS5A has a functional nuclear localization signal (NLS) at its C-terminal portion (aa 354 to 362) (14), but also possesses NLS-masking sequence at its N-terminus (31). NS5A mutants with an N-terminal deletion of 38 residues or more were demonstrated to be localized in the nucleus, supporting these findings (31). NS5A was shown to be cleaved by a caspase-like protease(s) at three sites, two of which have been mapped to positions 154 and 389 ($D^{154}$ and $D^{389}$) (26). NS5A was also reported to be cleaved at almost the same positions when the cells co-expressing HCV core underwent apoptosis (10). The cleavage product (aa 155 to 389) was localized in the nucleus and was shown to mediate transcriptional activation when expressed with protein kinase A (PKA) (26). There is also a report that NS5A was cleaved by calpain (15).

Caspases (cysteinyl aspartate-specific proteases) are a family of proteases that play an important role in apoptosis. Twelve mammalian caspases presently known are numbered in the chronological order of their identification. The caspase proteolytic signaling cascades are initiated by various external or internal signals and executed through several inter-related signaling pathways. Based on their proapoptotic functions, the caspases have been divided into two groups: initiators (caspase-2, -8, -9, -10 and probably, -11) and effectors (caspases-3, -6, and -7) (reviewed in 35).

In the present paper we report that NS5A was cleaved by caspase-3 under certain cellular condition(s) created by proapoptotic stimuli and ER stress. We have determined previously unknown, the third caspase-3 recognition/cleavage site ($D^{248}$-S-P-$D^{251}$) within ISDR of NS5A of some HCV-1b isolates. NS5A of other HCV isolates was not cleaved at $D^{251}$ by caspase-3 due to the mutation at position 248 whereas it was always cleavable at $D^{154}$ and $D^{389}$ due to the sequence conservation at the recognition/cleavage sites, T-E-L-$D^{154}$ and S-A-V-$D^{389}$.

## MATERIALS AND METHODS

**Plasmid construction.** Various plasmids expressing the entire NS5A tagged with the FLAG sequence (DYKDDDDK) at the N-terminus and c-Myc and 6× His at the C-terminus were constructed. To clarify the importance of aa sequence in the ISDR, three plasmids expressing NS5A of different strains of HCV-1b were subcloned by PCR amplification with appropriate sets of primers (Table 1) using pSGns5a-Jk, pSGns5a-94AJk and pSGns5a-J/AJ/J as templates (14), followed by digesting the amplified products with *Xba*I and *Hind*III and inserting into the unique *Xba*I-*Hind*III site of pcDNA3.1/Myc-His(-) (Invitrogen Corp., Carlsbad, CA, USA). The resultant plasmids were designated pcDNA-FLAG-ns5a-Jk-Myc-His, pcDNA-FLAG-ns5a-94AJk-Myc-His, pcDNA-FLAG-ns5a-J/AJ/J-Myc-His, respectively. Point mutations in the ISDR (at positions aa 248, 249 and 252) were introduced into these plasmids by site-directed mutagenesis. These expression plasmids were used for transient expression experiments using vaccinia virus-T7 hybrid system. FLAG-tagged NS5A sequence of the Jk strain was subcloned into the pCAGGS vector to generate pCAGGS-FLAG-ns5a-Jk for stable expression in cultured cells.

**Table 1. Primers used in this study.**

| Name | Polarity | Sequences |
|---|---|---|
| FLAG-NS5A-Jk-F | Sense | 5'-TATATCTAGA**ATG**GACTACAAAGACGATGACGACAAGATGTCCGGATCCTGGCTAAAGG-3' |
| FLAG-NS5A-AJk-F | Sense | 5'-TATATCTAGA**ATG**GACTACAAAGACGATGACGACAAGATGTCCGGATCCTGGCTCAGGG-3' |
| NS5A-R | Antisense | 5'-TATAAAGCTTTGCAGCAGACGACGTC-3' |

The translation initiation codons are shown in boldface letters.
The enzyme recognition sites are underlined.

**Transient expression of NS5A in FL, L929, HeLa and Huh7 cells.** FL human amnion cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 8% heat-inactivated calf serum. HeLa human cervical cells and Huh7 human hepatocarcinoma cells were maintained in DMEM with 10 % fetal calf serum (FCS). L929 mouse fibroblast cells were maintained in Eagle's minimum essential medium containing 8% FCS. All cells were kept at 37°C in a $CO_2$ incubator. Cells were transfected with either of the NS5A expression plasmids using FuGENE 6 (Roche Diagnostics) according to the manufacturer's protocol. Twenty four h after transfection, cells expressing NS5A were treated with cycloheximide (CHX; 10 μg/ml) and poly(I:C) (20 μg/ml) (Amersham Biosciences Corp., Piscataway, NJ, USA) for different periods of time as indicated in the figures.

**Protein detection and immunoblotting.** Cells were lysed in a buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 0.1% Triton X-100 and 0.5% sodium deoxycholate. Lysates were centrifuged at 15,000 rpm for 20 min at 4°C to clarify from cell debris, and supernatants were mixed with

gel-loading buffer (final concentration; 50 mM Tris-HCl (pH 6.8), 1% SDS, 5% N, N'-mercaptoethanol, 0.001% bromophenol blue and 2% glycerol) and were boiled for 5 min. The proteins were resolved in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were electrophoretically blotted onto a polyvinylidene difluoride filter (Millipore Corp., Bedford, MA, USA). The filters were blocked with 5% skim milk in phosphate buffered saline (PBS) and were incubated with an appropriate mouse monoclonal antibody (see below). After being washed with PBS containing 0.05% Tween 20, the filters were reacted with peroxidase-labeled goat anti-mouse IgG. The specific protein bands were visualized by an enhanced chemiluminescence method (ECL; Amersham Biosciences Corp.).

**Stable expression of NS5A in FL cells.** FL cells were co-transfected with a selection plasmid pSV2neo (0.1 μg) and either pCAGGS-FLAG-ns5A-Jk (2 μg) or the control pCAGGS vector (2 μg) by using FuGENE 6. After 2 to 3 weeks of selection in culture medium containing geneticin (G418; 400 μg/ml), the resultant colonies were collected without cell cloning, and the uncloned cells, respectively named FL/NS5A-Jk and FL/pCAG, were continuously maintained in medium containing 400 μg/ml geneticin. To clarify which caspase is responsible for NS5A cleavage, the stable FL transformants were treated with various caspase inhibitors at concentration of 25 μM 1 h before being treated with CHX and poly(I:C).

**Recombinant caspase-3 cleavage *in vitro*.** FL cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3). After 1 h, the cells were transfected with pcDNA-FLAG-ns5a-Jk-Myc-His, pcDNA-FLAG-ns5a-94AJk-Myc-His or pcDNA-FLAG-ns5a-J/AJ/J-Myc-His using Lipofectin reagent (Invitrogen, Inc.) according to the manufacturer's protocol. After cultivation for 16 h, cells were collected and lysed in a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA and 1% NP40. The cell lysates were clarified by centrifugation and incubated with anti-Myc antibody for 1 h at 4°C followed by addition of protein G Sepharose beads and incubation for another 30 min. After being washed with the same lysis buffer, the immunoprecipitates were treated with purified caspase-3 (Calbiochem-Novabiochem Corp.) for 3 h at 37°C in a buffer containing 62.5 mM Hepes-KOH (pH 7.4), 125 mM NaCl, 12.5 % glycerol and 25 mM DTT. The samples were then analyzed by immunoblotting with anti-FLAG M2 antibody.

**Antibodies and caspase inhibitors.** The primary antibodies used in immunoblotting and immunoprecipitation analysis were the following: (i) mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, Inc., St. Louis, MO, USA), (ii) mouse monoclonal anti-Myc antibody (sc-40; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and (iii) mouse monoclonal anti-6× His antibody (C-term; Invitrogen). The caspase inhibitors used in the analysis were: (i) caspase-1 inhibitor (Z-YVAD-FMK), (ii) caspase-2 inhibitor (Z-VDVAD-FMK), (iii) caspase-3 inhibitors (Z-DEVD-FMK and Z-DQMD-FMK), (iv) caspase-5 inhibitor (Z-WEHD-FMK), (v) caspase-6 inhibitor (Z-VEID-FMK), (vi) caspase-8 inhibitor (Z-IETD-FMK), (vii) caspase-9 inhibitor (Z-LEHD-FMK), and (viii) general caspase inhibitor (Z-VAD-FMK). All caspase inhibitors were purchased from Calbiochem-Novabiochem Corp., San Diego, CA, USA.

## RESULTS

**Cleavage of NS5A in cells treated with CHX and poly(I:C).** FL cells transiently expressing NS5A were treated with CHX and poly(I:C), both of which are known as apoptosis-inducing reagents. After being treated in various periods of time, cells were

collected, lysed and analyzed by immunoblotting. Three cleavage products of 48, 30 and 19 kDa were identified for NS5A of the HCV-Jk strain (Fig. 1A). The amounts of the latter two fragments (30 and 19 kDa) increased to accumulate until 6 h after treatment with CHX and poly(I:C) whereas the other fragment (48 kDa) became more apparent at 3 h but disappeared by 6 h post treatment. On the other hand, NS5A of the M094AJk strain generated only two cleavage products (48 and 19 kDa), with the 30-kDa-fragment being missing. The different cleavage patterns between the two HCV strains were consistently observed in other cell lines, such as L929, HeLa and Huh7 (data not shown). From these results, we concluded that the 19 kDa and 48 kDa fragments were those cleaved at $D^{154}$ and $D^{389}$, respectively, as reported by Satoh *et al*. (26), and that the disappearance of the 48 kDa fragment 6 h after the treatment, specifically observed for NS5A-Jk, was due to the cleavage somewhere within or around the ISDR, which generated the 30 kDa fragment. When cells were preincubated with Z-VAD-FMK, a general caspase inhibitor, the NS5A cleavage was almost completely suppressed. Immunoblot analysis of the cell lysates using anti-Myc (Fig. 1B) and anti-6×His antibodies (data not shown) detected only the full-size NS5A, suggesting that the cleavage at $D^{389}$ occurs first, leaving the N-terminal fragments undetectable by these antibodies. The Myc-His-tagged, short C-terminal fragment (aa 390 to 447) with the expected size of ~8 kDa was likely to have passed through the gel in Fig. 1B.

**Inhibition of the NS5A cleavage by various caspase inhibitors.** To determine which caspase(s) is responsible for the cleavage of NS5A, we used a series of caspase inhibitors. When FL cells stably expressing NS5A (FL/NS5A-Jk) were treated with CHX and poly(I:C) without caspase inhibitor pretreatment, NS5A was cleaved to generate three fragments of 48, 30 and 19 kDa (Fig. 2A), with the pattern being practically the same as that observed in Fig. 1A. Pretreatment of the cells with caspase-2 or -5 tetrapeptide inhibitors did not change the NS5A cleavage pattern. However, when treated with either caspase-1, -3, -6, -8 or -9 inhibitor, NS5A cleavage was almost completely inhibited. As caspase-3 is the most downstream effector of those 5 caspases (-1, -3, -6, -8 and -9), we suspected that the cleavage was carried out by caspase-3. We confirmed that the inhibition of NS5A cleavage by a more specific caspase-3 inhibitor (Z-DQMD-FMK) and the inhibition was dose-dependent (Fig. 2B). These results collectively suggest that caspase-3 is principally responsible for the NS5A cleavage.

**Cleavage of NS5A by recombinant caspase-3 *in vitro*.** To directly assess the involvement of caspase-3 in NS5A cleavage, we performed an *in vitro* cleavage experiment. NS5A transiently expressed in FL cells using vaccinia expression system was immunoprecipitated using anti-Myc antibody, treated with purified recombinant caspase-3 and analyzed by immunoblotting with anti-FLAG antibody. As shown in Fig. 3, NS5A of the Jk strain was cleaved *in vitro* to generate three fragments of 48 kDa (corresponding to aa 1 to 389), 30 kDa (aa 1 to 251; see below) and 19 kDa (aa 1 to 154). On the other hand, NS5A of the M094AJk strain (94AJk) and a chimeric NS5A (J/AJ/Jk), whose ISDR was derived from the M094AJk strain, were cleaved to generate only two fragments of 48 and 19 kDa. These cleavage patterns *in vitro* are consistent with the patterns of intracellular cleavage of NS5A (see Fig. 1A). Also, this result suggests the possible presence of a cleavage site in ISDR of NS5A-Jk.

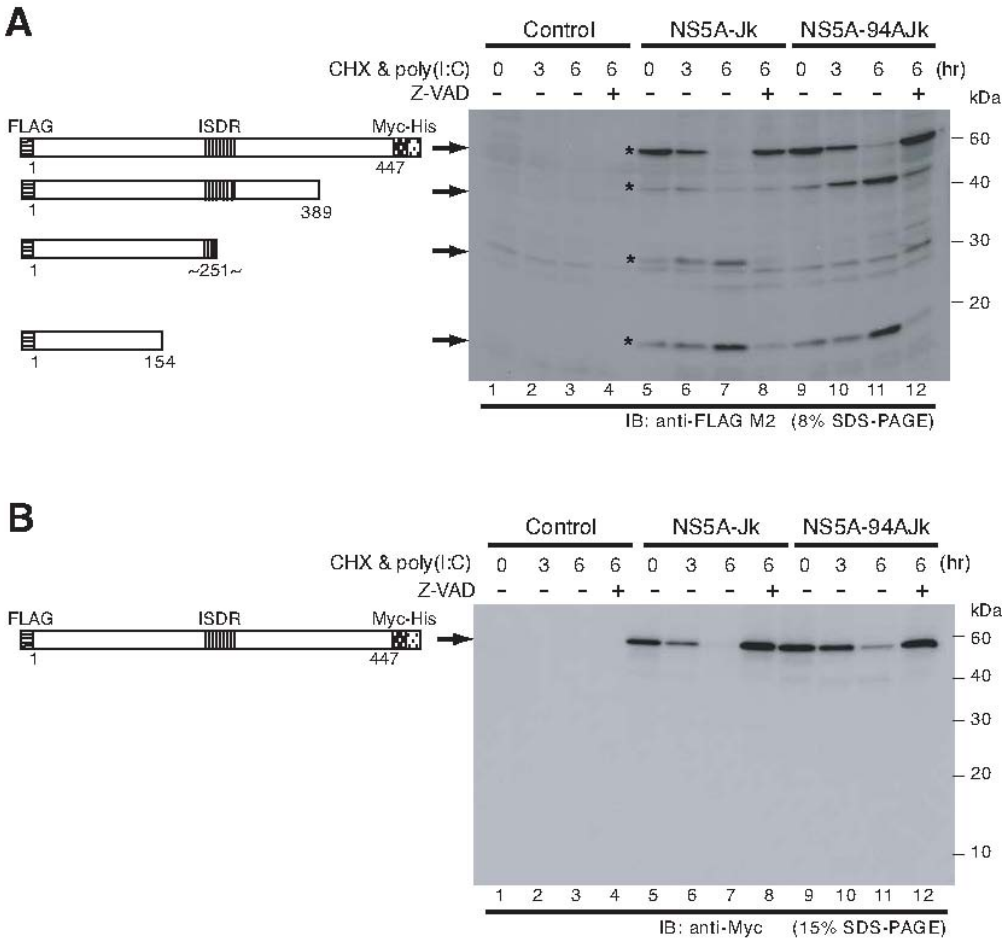


**FIG. 1.** NS5A cleavage and its inhibition by a general caspase inhibitor in cells treated with CHX and poly(I:C). FL cells transiently transfected with a control plasmid, pcDNA3.1/Myc-His(-) (Control, lanes 1 to 4), pcDNA-FLAG-ns5a-Jk-Myc-His (NS5A-J, lanes 5 to 8) or pcDNA-FLAG-ns5a-94AJk-Myc-His (NS5A-AJ, lanes 9 to 12) were pretreated with (+; lanes 4, 8 and 12) or without 40 μM Z-VAD-FMK (–; lanes 1 to 3, 5 to 7, and 9 to 11) for 1 h, and then treated with CHX (10 μg/ml) and poly(I:C) (20 μg/ml) for the indicated time period. Cell lysates were resolved in 8 % gel SDS-PAGE and subjected to immunoblotting analysis using anti-FLAG M2 antibody (A) or in 15 % gel SDS-PAGE and reacted with anti-Myc antibody (B). Asterisks indicate the positions of uncleaved and cleaved NS5A bands. Schematic representation of full-length NS5A and its cleaved products tagged with Myc-His and/or FLAG is shown on the left. Numbers under the bars denote aa positions of NS5A. Molecular mass markers in kDa are indicated on the right.

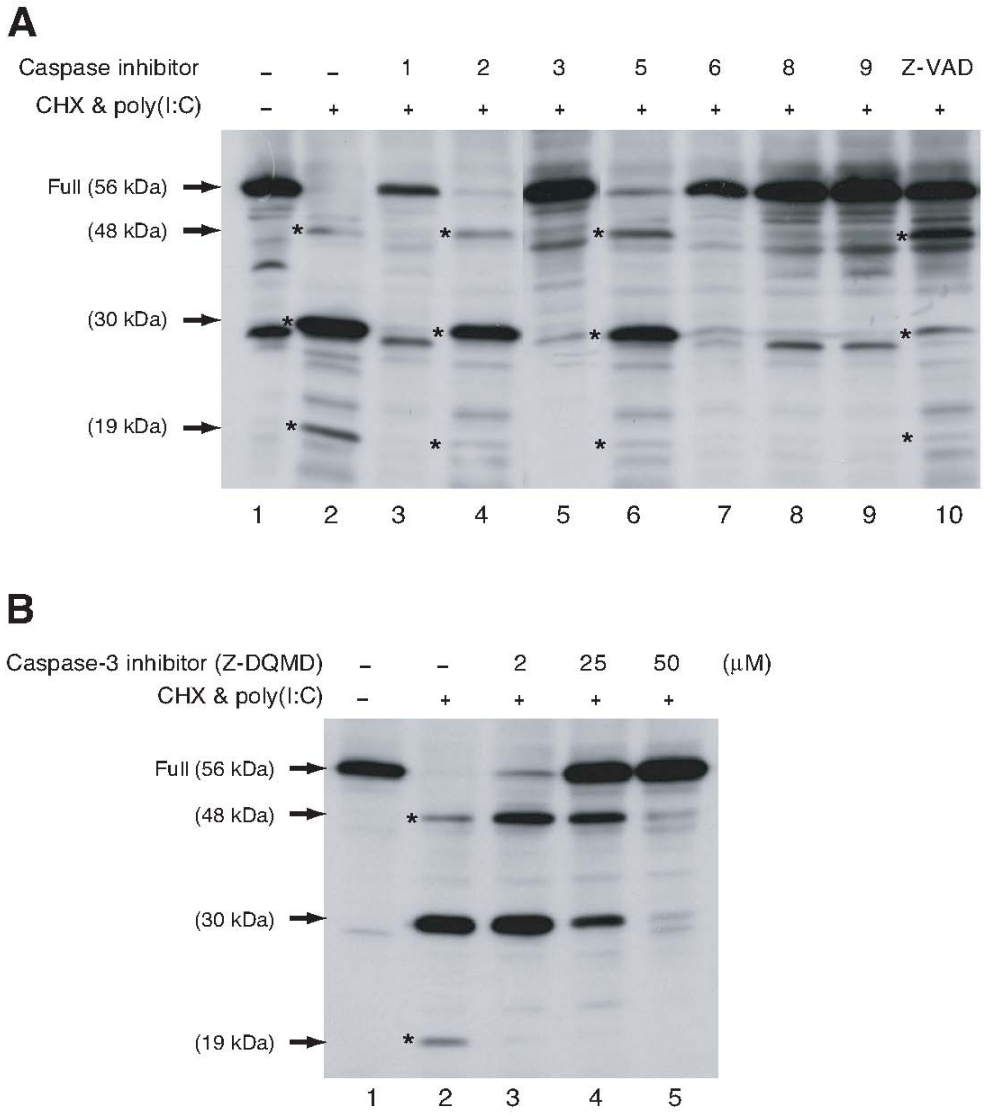


**FIG. 2.** Inhibition of NS5A cleavage by caspase inhibitors. (A) FL cells stably expressing NS5A were pretreated with various caspase inhibitors, as indicated on the top, before being treated with CHX (10 μg/ml) and poly(I:C) (20 μg/ml). Cell lysates were subjected to immunoblotting analysis using anti-FLAG antibody. Asterisks indicate the position of the expected bands. (B) FL cells stably expressing NS5A were pretreated with caspase-3 inhibitor Z-DQMD-FMK at the indicated concentration, before being treated with CHX (10 μg/ml) and poly(I:C) (20 μg/ml).

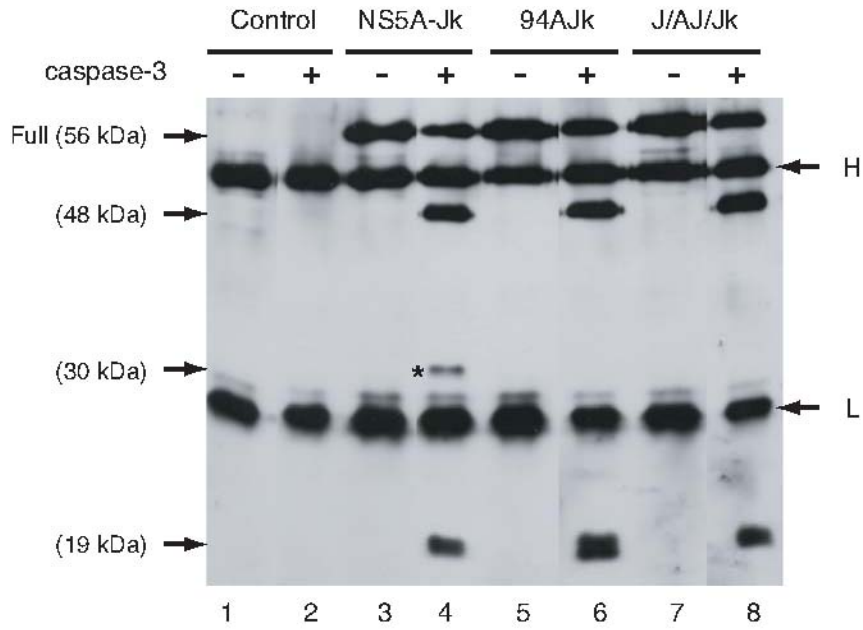


**FIG. 3**.
In vitro cleavage of NS5A by recombinant caspase-3. Full-length FLAG-NS5A-Myc-His immunoprecipitated with anti-Myc antibody was incubated with recombinant caspase-3 at 37°C for 3 h, and subsequently analyzed by immunoblotting with anti-FLAG antibody. Asterisk indicates the position of the 30kDa band of cleaved NS5A-Jk. H, IgG heavy chain; L, IgG light chain.

**Determination of the cleavage site in ISDR by site-directed mutagenesis.** The consensus sequence(s) of the caspase-3 cleavage site has been reported to be D-X-X-D (3,13,18,20,33). Some noncanonical sequences, such as S-A-L-D and V-E-V-D, were also reported (17,19). Previously identified two caspase recognition sites in NS5A (T-E-L-$D^{154}$ and S-A-V-$D^{389}$) are also noncanonical. As shown above, a third recognition site for caspase-3 was expected to be present in ISDR of the Jk strain, but not in that of the M094AJk strain. There are two potential caspase-3 recognition sites in and around ISDR of the Jk strain, $D^{248}$-S-P-$D^{251}$ and $D^{282}$-S-F-$D^{285}$ (Fig. 4). Considering the size of the cleavage product of 30 kDa, we focused on the $D^{248}$-S-P-$D^{251}$ sequence. As $D^{251}$ is shared by the Jk and M094AJk strains (and also by the chimeric NS5A), and as $G^{248}$ replaces $D^{248}$ in the M094AJk strain and the chimeric NS5A (see Fig. 5A), we introduced a point mutation at position 248 of the Jk strain so that the D-X-X-D consensus sequence was no longer exist. The introduction of the D-to-G single-point mutation at this position to NS5A of the Jk strain completely abolished the generation of 30-kDa-fragment (Fig. 5B, lane 5). In order to see whether or not the combination of $D^{248}$ and $D^{251}$ was sufficient for the caspase recognition and cleavage in the context of the NS5A sequence, we introduced a G-to-D mutation at position 248 to the chimeric NS5A-J/AJ/Jk and cleavability of this NS5A mutant was examined. The result showed that the 30-kDa-cleavage product was detected only faintly (lane 8). This suggests that $D^{248}$ and $D^{251}$ alone are not sufficient for complete recognition

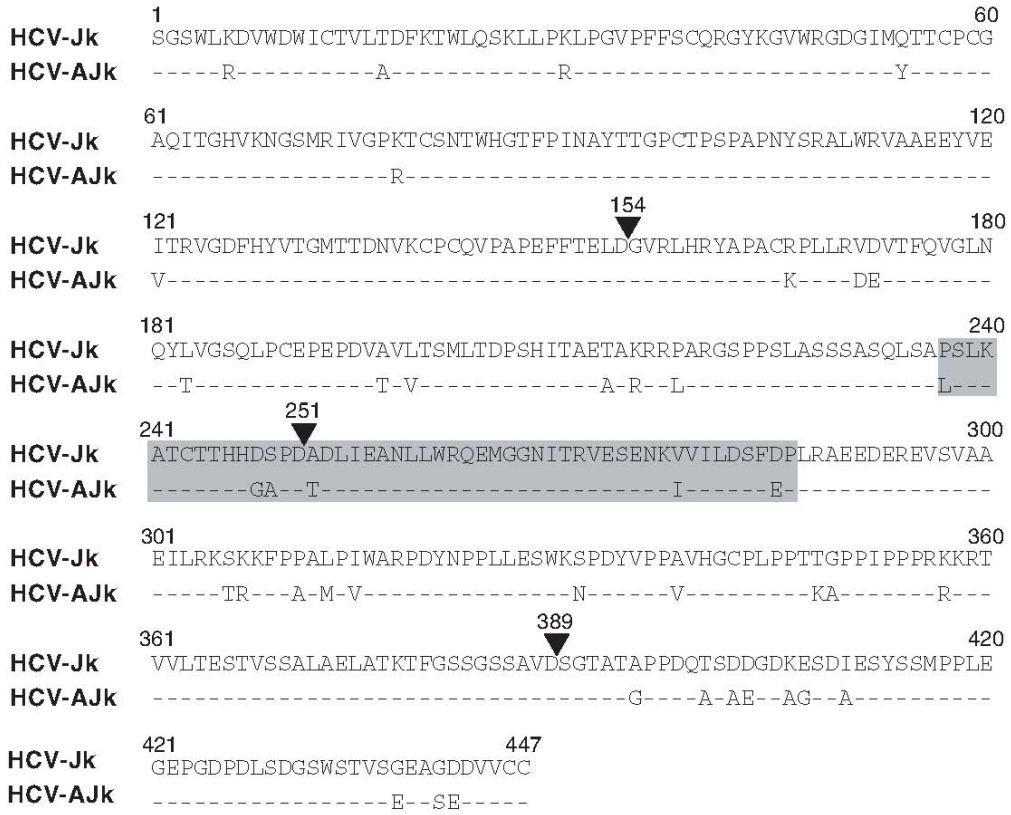


**Fig. 4.** Alignment of aa sequences of NS5A of the HCV-Jk and M094AJk strains. Hyphens indicate residues identical to those of the HCV-Jk sequence. Arrow heads indicate the putative caspase recognition/cleavage sites. The chimeric portion including ISDR sequence is enclosed in a shaded box (see Fig. 5A).

and/or cleavage by caspase-3. The 30-kDa-fragment was still faint when an additional mutation was introduced at position 249 (lane 9). The introduction of a further mutation at position 252, which makes the sequence of this site completely match that of the Jk strain, resulted in more efficient cleavage of NS5A (lane 10). Taken together, these results suggest the possibility that $D^{248}$-S-P-$D^{251}$-$A^{252}$ is required for full recognition and/or cleavage by caspase-3 in the context of NS5A sequence.

**NS5A cleavage in the absence of CHX and poly(I:C) treatment.** It is known that expression of a particular viral protein(s) itself as well as virus replication in the cell triggers apoptotic pathways. Therefore, we were interested to know whether NS5A cleavage occurs even without CHX and poly(I:C) treatment. In this experiment, FL cells transiently expressing NS5A were first treated with a general caspase inhibitor Z-VAD-FMK for 18 h. As had been expected, NS5A cleavage was completely inhibited by Z-VAD-FMK (Fig. 6, lane 1). Six hours after removal of Z-VAD-FMK, however, three cleavage products of 48, 30 and 19 kDa became detectable (lane 2). Continuous treatment with Z-VAD-FMK still successfully inhibited the NS5A cleavage (lane 3). These results suggest that NS5A cleavage occurs even in the absence of external proapoptotic stimuli.

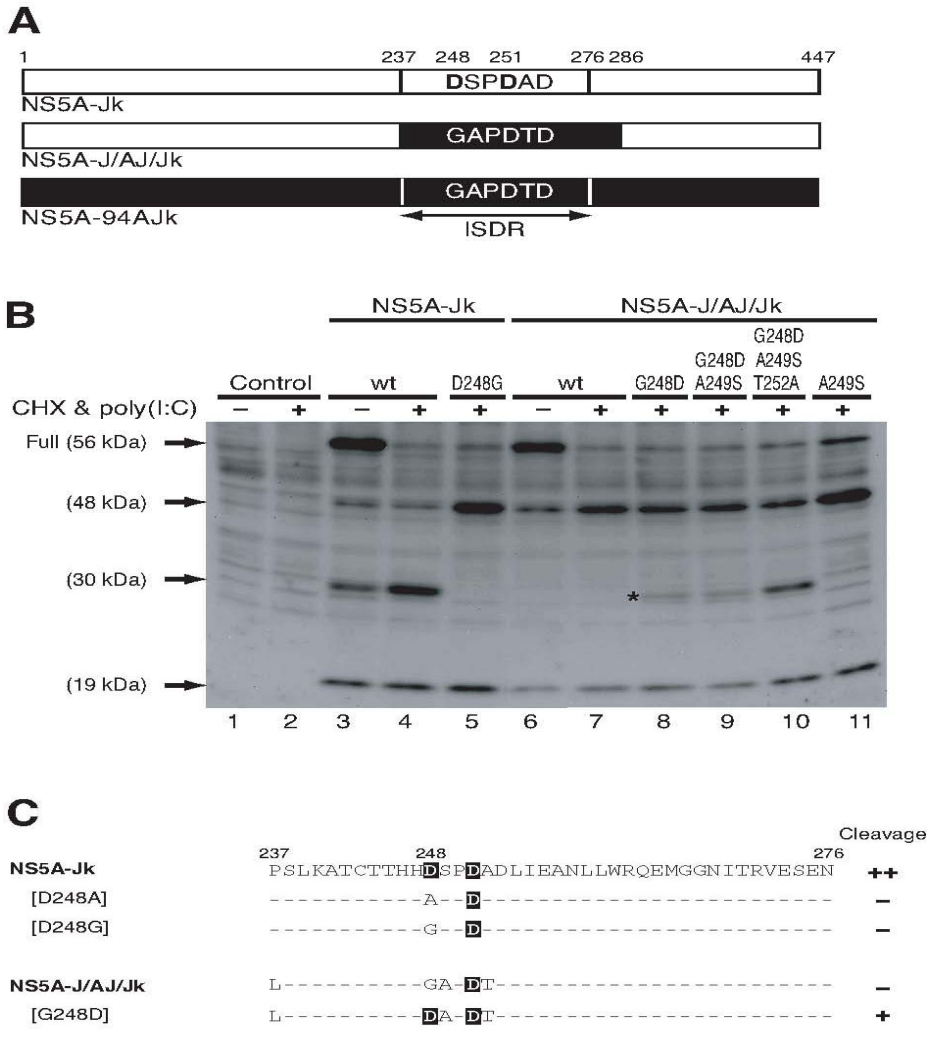


**FIG. 5.** Effects of aa mutations at the putative caspase-3 recognition/cleavage site in ISDR. (A) Schematic representation of NS5A of the Jk and M094AJk strains and their chimeric form. The sequences of HCV-Jk and M094AJk are depicted by white and black rectangles, respectively. The numbers indicate the aa positions of NS5A. (B) FL cells transiently expressing full-length FLAG-tagged NS5As and their mutants were treated with (lanes 2, 4, 5, and 7 to 11) or without (lanes 1, 3 and 6) 10 µg/ml CHX and 20 µg/ml poly(I:C) for 3 h. Cell lysates were subjected to immunoblotting analysis using anti-FLAG M2 antibody. Mutated aa residues are indicated on the top of the lanes. Asterisks indicate the position of the expected bands. (C) Amino acid sequence alignment of NS5A-Jk, NS5A-J/AJ/Jk, and their mutants. Hyphens represent residues identical to those of HCV-Jk sequence. White letters in black box indicate aa residues important for efficient cleavage. ++, strong; +, weak; –, no cleavage.

## DISCUSSION

In the present study, we showed the evidence that HCV NS5A was cleaved into two (48 and 19 kDa) or three fragments (48, 30 and 19 kDa), depending upon the HCV isolates tested, when cells received proapoptotic stimuli such as CHX and poly(I:C) treatment (Fig. 1). We also demonstrated that caspase-3 was the principal enzyme responsible for the cleavage (Figs. 2 and 3). The fragments of 48 and 19 kDa were likely to result from the cleavage at the previously identified two sites, $D^{154}$ and $D^{389}$ (26), while the third cleavage site responsible for the generation of the 30 kDa fragment was as-yet-undetermined. In this study we have mapped the third cleavage site to position 251 ($D^{251}$) within ISDR of NS5A (Fig. 5). Although $D^{251}$ was highly conserved among HCV-1b clinical isolates, the consensus caspase-3 recognition sequence ($D^{248}$-X-X-$D^{251}$) is not conserved due to the sequence variation at

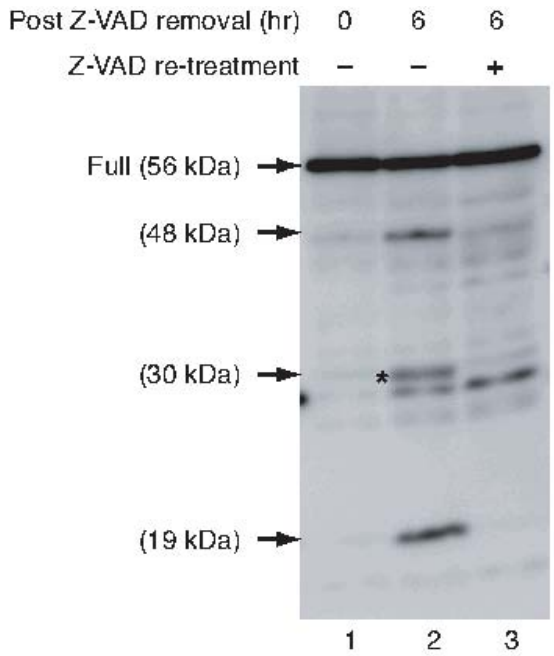


**FIG. 6.** NS5A cleavage in the transient expression system without CHX and poly(I:C) treatment. FL cells transiently expressing FLAG-ns5a-Jk-Myc-His were treated with 40 μM Z-VAD-FMK for 18 h. Z-VAD-FMK was then removed by washing the cells several times with culture medium. The cells were collected (lane 1) and further cultured for 6 h with (lane 2) or without (lane 3) Z-VAD-FMK retreatment and subjected to immunoblotting analysis using anti-FLAG M2 antibody.

position 248 in ISDR. Furthermore, the residue ($A^{252}$) next to the $D^{248}$-X-X-$D^{251}$ consensus sequence which also varies with different HCV isolates, is likely to be involved in the full recognition by caspase-3 (Fig. 5). These sequence variations well explain the isolate-dependent NS5A cleavage at $D^{251}$ that generates the 30-kDa-fragment. The present result also suggests the possibility that the cleavage at $D^{389}$ precedes that at $D^{154}$ and $D^{251}$ (Fig. 1B).

Caspase-mediated processing of NS5A generates both N- and C-terminally deleted forms. Although the virological significance of the NS5A processing by caspases is still unclear, Satoh *et al*. (26) reported that one of the cleavage products (aa 154-389) had transcriptional activity in cooperation with PKA. The NS5A sequence used in their study has the $D^{248}$-S-P-$D^{251}$-$A^{252}$ sequence and, therefore, is expected to be cleaved by caspase-3. It is intriguing to speculate that NS5A of other strains that do not have the consensus $D^{248}$-S-P-$D^{251}$-$A^{252}$

sequences might exert a stronger transcriptional activity. It is thus possible that transcriptional activity of NS5A differs with different HCV strains. In fact, Fukuma *et al.* (7) reported using yeast and mammalian cell systems that transcriptional activity was enhanced by aa mutations in ISDR, which are associated with decreased viral load and increased IFN sensitivity. Another possibility should also be taken into consideration that NS5A competes with the cellular substrates of caspase-3, such as poly ADP-ribose polymerase that cleaves DNA into fragments, thereby preventing the cell from apoptosis. In this connection, it was reported that, despite an increased caspase-3 activity in the cells, majority of hepatocytes in HCV-infected patients did not undergo apoptosis, as demonstrated by the lack of TUNEL reactivity (2).

We observed NS5A cleavage in cells transiently expressing NS5A even in the absence of CHX and poly(I:C) treatment (Fig. 6). On the other hand, in cells stably expressing NS5A, the cleavage was not observed in the absence of CHX and poly(I:C) treatment (see Fig. 2). In general, transient expression of a protein is thought to induce stronger ER stress, which activates caspase-3, than that induced by stable, weaker expression of the same protein (11). Also, replication of HCV subgenomic RNA replicon was reported to induce ER stress (32). It is thus possible that NS5A cleavage occurs in HCV subgenomic RNA replicon-harboring cells and also in hepatocytes of HCV-infected patients. Further study is needed to elucidate the issues.

## ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS) and a research grant from Research Foundation of Viral Hepatitis, Japan.

## REFERENCES

1. **Apichartpiyakul, C., C. Chittivudikarn, H. Miyajima, M. Homma, and H. Hotta.** 1994. Analysis of hepatitis C virus isolates among healthy blood donors and drug addicts in Chiang Mai, Thailand. J. Clin. Microbiol. **32**:2276-2279.
2. **Bantel, H., A. Lugering, C. Poremba, N. Lugering, J. Held, W. Domschke, and K. Schulze-Osthoff.** 2001. Caspase activation correlates with the degree of inflammatory liver injury in chronic hepatitis C virus infection. Hepatology **34**:758-67.
3. **Brancolini, C., D. Lazarevic, J. Rodriguez, and C. Schneider.** 1997. Dismantling cell-cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of beta-catenin. J. Cell. Biol. **139**:759-771.
4. **Doi, H., C. Apichartpiyakul, K. Ohba, M. Mizokami, and H. Hotta.** 1996. Hepatitis C virus (HCV) subtype prevalence in Chiang Mai, Thailand, and identification of novel subtypes of HCV major type 6. J. Clin. Microbiol. **34**:569-574.
5. **Enomoto, N., I. Sakuma, Y. Asahina, M. Kurosaki, T. Murakami, C. Yamamoto, N. Izumi, F. Marumo, and C. Sato.** 1995. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J. Clin. Invest. **96**:224-230.
6. **Enomoto, N., I. Sakuma, Y. Asahina, M. Kurosaki, T. Murakami, C. Yamamoto, Y. Ogura, N. Izumi, F. Marumo, and C. Sato.** 1996. Mutations in the nonstructural 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection.

N. Engl. J. Med. **334**:77-81.

7. **Fukuma, T., N. Enomoto, F. Marumo, and C. Sato.** 1998. Mutations in the interferon-sensitivity determining region of hepatitis C virus and transcriptional activity of the nonstructural region 5A protein. Hepatology **28**:1147-1153.
8. **Gale, M. Jr., C.M. Blakely, B. Kwieciszewski, S.L. Tan, M. Dossett, N.M. Tang, M.J. Korth, S.J. Polyak, D.R. Gretch, and M.G. Katze.** 1998. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: Molecular mechanisms of kinase regulation. Mol. Cell. Biol. **18**:5208-5218.
9. **Gerotto, M., F. Dal Pero, P. Pontisso, F. Noventa, A. Gatta, and A. Alberti.** 2000. Two PKR inhibitor HCV proteins correlate with early but not sustained response to interferon. Gastroenterology **119**:1649-1655.
10. **Goh, P.Y., Y.J. Tan, S.P. Lim, S.G. Lim, Y.H. Tan, and W.J. Hong.** 2001. The hepatitis C virus core protein interacts with NS5A and activates its caspase-mediated proteolytic cleavage. Virology **290**:224-236.
11. **Hitomi, J., T. Katayama, M. Taniguchi, A. Honda, K. Imaizumi, and M. Tohyama.** 2004. Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12. Neurosci. Lett. **357**:127-130.
12. **Hofgartner, W.T., S.J. Polyak, D.G. Sullivan, R.L. Carithers Jr., and D.R. Gretch.** 1997. Mutation in the NS5A gene of hepatitis C virus in North American patients infected with HCV genotype 1a or 1b. J. Med. Virol. **53**:118-126.
13. **Hofmann, T.G., S.P. Hehner, W. Droge, and M.L. Schmitz.** 2000. Caspase-dependent cleavage and inactivation of the Vav1 proto-oncogene product during apoptosis prevents IL-2 transcription. Oncogene **19**:1153-1163.
14. **Ide, Y., L. Zhang, M. Chen, G. Inchauspe, C. Bahl, Y. Sasaguri, and R. Padmanabhan.** 1996. Characterization of the nuclear localization signal and subcellular distribution of hepatitis C virus nonstructural protein NS5A. Gene **182**:203-211.
15. **Kalamvoki, M., and P. Mavromara.** 2004. Calcium-dependent calpain proteases are implicated in processing of the hepatitis C virus NS5A protein. J. Virol. **78**:11865-11878.
16. **Kaneko, T., Y. Tanji, S, Satoh, M, Hjikata, S. Asabe, K. Kimura, and K. Shimotohno.** 1994. Production of two phosphoproteins from the NS5A region of the hepatitis C viral genome. Biochem. Biophys. Res. Commun. **205**:320-326.
17. **Kipp, M., B.L. Schwab, M. Przybylski, P. Nicotera, and F.O. Fackelmayer.** 2000. Apoptotic cleavage of scaffold attachment factor A (SAF-A) by caspase-3 occurs at a noncanonical cleavage site. J. Biol. Chem. **275**:5031-5036.
18. **Kook, S., S.R. Shim, S.J. Choi, J. Ahnn, J.I. Kim, S.H. Eom, Y.K. Jung, S.G. Paik, and W.K. Song.** 2000. Caspase-mediated cleavage of p130cas in etoposide-induced apoptotic Rat-1 cells. Mol. Biol. Cell. **11**:929-939.
19. **Ku, N.O., and M.B. Omary.** 2001. Effect of mutation and phosphorylation of type I keratins on their caspase-mediated degradation. J. Biol. Chem. **276**:26792-26798.
20. **Liu, F., M. Dowling, X.J. Yang, and G.D. Kao.** 2004. Caspase-mediated specific cleavage of human histone deacetylase 4. J. Biol. Chem. **279**:34537-34546.
21. **Major, M.E., B. Rehermann, and S.M. Freinstone.** 2001. Hepatitis C viruses, p. 1127-1161. In D.M. Knipe and P.M. Howley (ed.). Fields Virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
22. **Mellor, J., E.C. Holmes, L.M. Jarvir, P.L. Yap, and P. Simmonds.** 1995.

Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. J. Gen. Virol. **76**:2493-2507.

23. **Pawlotsky, J.M., G. Germanidis, A.U. Neumann, M. Pellerin, P.O. Frainais, and D. Dhumeaux.** 1998. Interferon resistance of hepatitis C virus genotype 1b: Relationship to nonstructural 5A gene quasispecies mutations. J. Virol. **72**:2795-2805.
24. **Robertson, B., G. Myers, C. Howard, T. Brettin, J. Bukh, B. Gashen, T. Gojobori, G. Maertens, M. Mizokami, O. Nainan, S. Netesov, K. Nishioka, T. Shin-i, P. Simmonds, D. Smith, L. Stuyver, and A. Weiner.** 1998. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related virus: proposals for standardization. Arch. Virol. **143**:2493-2503.
25. **Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Q.-L. Choo, M. Houghton, and G. Kuo.** 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. U.S.A. **87**:6547-6549.
26. **Satoh, S., M. Hirota, T. Noguchi, M. Hijikata, H. Handa, and K. Shimotohno.** 2000. Cleavage of hepatitis C virus nonstructural protein 5A by a caspase-like protease(s) in mammalian cells. Virology **270**:476-487.
27. **Schafer, D.F., and M.F. Sorrell.** 1999. Hepatocellular carcinoma. Lancet **353**:1253-1257.
28. **Simonetti, R.G., C, Camma, F. Fiorello, M. Cottane, M. Rapicetta, L. Marino, G. Fiorentino, A. Craxi, A. Ciccaglione, R. Giuseppetti, T. Stroffolini, and L. Pagliaro.** 1992. Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. A case-control study. Ann. Intern. Med. **116**:97-102.
29. **Soetjipto, R. Handajani, M.I. Lusida, S. Darmadi, P. Adi, Soemarto, S. Ishido, Y. Katayama, and H. Hotta.** 1996. Differential prevalence of hepatitis C virus subtypes in healthy blood donors, patients on maintenance hemodialysis, and patients with hepatocellular carcinoma in Surabaya, Indonesia. J. Clin. Microbiol. **34**:2875-2880.
30. **Song, J., M. Fujii, F. Wang, M. Itoh, and H. Hotta.** 1999. The NS5A protein of hepatitis C virus partially inhibits the antiviral activity of interferon. J. Gen. Virol. **80**:879-886.
31. **Song, J., M. Nagano-Fujii, F. Wang, R. Florese, T. Fujita, S. Ishido, and H. Hotta.** 2000. Nuclear localization and intramolecular cleavage of N-terminally deleted NS5A protein of hepatitis C virus. Virus Res. **69**:109-117.
32. **Tardif, K.D., K. Mori, R.J. Kaufman, and A. Siddiqui. 2**004. Hepatitis C virus suppresses the IRE1-XBP1 pathway of the unfolded protein response. J. Biol. Chem. **279**:17158-17164.
33. **Tu, S., and R.A. Cerione.** 2001. Cdc42 is a substrate for caspases and influences Fas-induced apoptosis. J. Biol. Chem. **276**:19656-19663.
34. **Witherell, G.W., and P. Beineke.** 2001. Statistical analysis of combined substitutions in nonstructural 5A region of hepatitis C virus and interferon response. J. Med. Virol. **63**:8-16.
35. **Wolf, B.B., and D.R. Green.** 1999. Suicidal tendencies: apoptotic cell death by caspase family proteinases. J. Biol. Chem. **274**:20049-20052.