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Hepatitis C Virus-Encoded Nonstructural Protein NS4A Has Versatile Functions in Viral Protein Processing

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A transient protein expression system in COS-1 cells was used to study the role of hepatitis C virus (HCV)-encoded NS4A protein on HCV nonstructural polyprotein processing. By analyzing the protein expression and processing of a deletion mutant polypeptide, NSΔ4A, which encodes the entire putative HCV nonstructural polyprotein except the region encoding NS4A, the versatile functions of NS4A were revealed. Most of the NS3 processed from NSΔ4A was localized in the cytosol fraction and was degraded promptly. Coproduction of NS4A stabilizes NS3 and assists in its localization in the membrane. NS4A was found to be indispensable for cleavage at the 4B/5A site but not essential for cleavage at the 5A/5B site in NSΔ4A. The functioning of NS4A as a cofactor for cleavage at the 4B/5A site was also observed when 30 amino acids around this site was used as a substrate and a serine proteinase domain of 167 amino acids, from Gly-1049 to Ser-1215, was used as an enzyme protein, suggesting that possible domains for the interaction of NS4A were in those regions of the enzyme protein (NS3) and/or the substrate protein. Two proteins, p58 and p56, were produced from NS5A. For the production of p58, equal or excess molar amounts of NS4A relative to NSΔ4A were required. Deletion analysis of NS4A revealed a minimum functional domain of NS4A of 10 amino acids, from Gly-1678 to Ile-1687.

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, has a positive-stranded RNA of about 9.5 kb (5, 13, 19). The precursor polyprotein translated from the longest open reading frame of the genome undergoes proteolytic processing conducted by both host and viral proteinases (2, 4, 6, 9–12, 15, 16, 22). For HCV type 1b (HCV-1b), the major genotype in Japan (13), the order of the individual proteins on the viral genome is NH_2-C (p22)-E1 (gp35)-E2 (gp70)-NS2 (p21)-NS3 (p70)-NS4A (p4)-NS4B (p27)-NS5A (p58/p56)-NS5B (p66)-COOH, where C, E, and NS are the putative core, envelope, and nonstructural proteins, respectively (11). A viral serine proteinase, Cpro-2, located in the N-terminal part of NS3 is employed for processing the region downstream of NS3 by two different modes of cleavage, intramolecular (*cis*) and intermolecular (*trans*) proteolytic cleavages. The NS3/NS4A (abbreviated 3/4A) junction is processed only in a *cis* cleavage manner, whereas NS4A/NS4B (4A/4B), NS4B/NS5A (4B/5A), and NS5A/NS5B (5A/5B) junctions are cleaved in a *trans* cleavage manner (20, 22). The Cpro-2 domain which is necessary and sufficient for the cleavage at the 5A/5B site maps from Gly-1049 to Ser-1215 of the HCV precursor polyprotein (20).

In vitro transcription and translation analysis of the HCV nonstructural polyprotein showed that NS4A helps to anchor NS3 to the surface of the microsomal membrane by mutual association of these two proteins (12). Furthermore, the NS3-NS4A complex associates with NS4B, NS5A, and NS5B to form a complex structure which may be involved in virus replication. These observations may imply that NS4A plays an important role in complex formation. Recently, it was reported that NS4A is necessary for cleavage at the 4B/5A site and that it enhances cleavage at NS3-dependent cleavage sites (3, 7).

For this report, we analyzed the processing of a mutated NSΔ4A polypeptide which encodes the entire putative HCV nonstructural polyprotein except the region of NS4A. Our experiments revealed versatile functions of NS4A. NS4A acts not only in the cleavage at the 4B/5A site but also in the stabilization and localization of NS3. A stoichiometrical assay and deletion analysis were also carried out to reveal the functions of NS4A.

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MATERIALS AND METHODS

Construction of expression plasmids. The construction of plasmids pCMV/N729-3010 and pCMV/N1049-1215 has been described previously (11, 20). Combinations of positive- and a negative-stranded primers used for PCR (described below) are indicated in parentheses after the PCR products. The oligonucleotides used in the constructions are summarized in Table 1. To obtain plasmid pCMV/NSΔ4A, which encodes the entire HCV nonstructural polyprotein except NS4A, the *Bam*HI-*Pst*I fragment of pCMV/N729-3010 was inserted into the *Bam*HI-*Pst*I site of pUC18 and used as a template for making PCR product 1 (F1 and M13 reverse primer) and PCR product 2 (M13 universal primer and R1). Both PCR products were ligated after phosphorylation, digested with *Bam*HI and *Pst*I, and then inserted into the *Bam*HI-*Pst*I site of pUC18. The resultant plasmid was pUC18/NSΔ4A, which carries the *Bam*HI-*Pst*I fragment of pCMV/N729-3010 except for the region encoding NS4A. pCMV/NSΔ4A was obtained by replacing the *Bam*HI-*Pst*I fragment of pCMV/N729-3010 with the *Bam*HI-*Pst*I fragment of pUC18/NSΔ4A. PCR product 3 (F2 and R2) was digested with *Pst*I-*Eco*RI and inserted into the *Pst*I-*Eco*RI site of pKS(+)/CMV (11) to obtain pCMV/N1658-1711, which encodes NS4A. To obtain plasmids that produce proteins with 30 amino acids (aa) around the 4B/5A or 5A/5B cleavage sites fused in frame to their C termini by an *Escherichia coli* dihydrofolate reductase (DHFR), PCR products 4 (F3 and R3) and 5 (F4 and R4) were digested with *Pst*I and *Hin*dIII and were replaced with the *Pst*I-*Hin*dIII fragment of pCMV/N729-1233D (20). The resultant plasmids were pCMV/N1953-1982D and pCMV/N2400-2429D, respectively. Deletion mutants of NS4A were constructed by replacing the *Pst*I-*Eco*RI fragments of PCR products 6 (F5 and R2), 7 (F6 and R2), 8 (F7 and R2), 9 (F2 and R5), 10 (F2 and R6), and 11 (F2 and R7) with the *Pst*I-*Eco*RI fragment of pCMV/N1658-1711. The resultant plasmids were pCMV/N1668-1711, pCMV/N1678-1711, pCMV/N1688-1711, pCMV/N1658-1697, pCMV/N1658-1687, and pCMV/N1658-1677, respectively.

Expression of HCV polyproteins in COS-1 cells. DNA transfections were performed as described previously (10). Lysates of COS-1 cells transfected with the series of pCMV-derived plasmids were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot analysis as described previously (10). The antibodies used in this experiment were anti-NS3 antibody (α-NS3), anti-NS4A antibody (α-NS4A), anti-NS4B

TABLE 1. Oligonucleotide primers for the PCR used in the construction of the pCMV plasmids

Designation	Nucleotide sequence[a]	Amino acids[b]
F1	5′-GCCTCACACCTCCCTTACATC-3′	1712–1718
F2	5′-GTCTGCAGATGAGCACCTGGGTGCTG-3′	1658–1662
F3	5′-TCGACTGCAGCCATGATCACTCAGCTGCTGAAG-3′	1953–1958
F4	5′-TCGACTGCAGCCATGCTCAGCGACGGGTCTTGG-3′	2400–2405
F5	5′-GACTGCAGATGGCAGCTCTGGCCGCA-3′	1668–1672
F6	5′-GCCTGCAGATGGGTAGTGTGGTCATT-3′	1678–1682
F7	5′-ATCTGCAGATGTTGTCCGGGAGGCCG-3′	1688–1692
R1	5′-AGTGACGACCTCCAGGTCGGC-3′	1657–1651
R2	5′-TCGAATTCTTAGCACGCTTCCATTTC-3′	1711–1707
R3	5′-CCGAAGCTTGTCCCAAACATCCCTTAG-3′	1982–1977
R4	5′-CCGAAGCTTCAAGGCACCTGTCCATGT-3′	2429–2424
R5	5′-ATGAATTCTTAGTCGGGAACAACAGC-3′	1697–1693
R6	5′-CAGAATTCTTAAATGATCCTACCCAC-3′	1687–1683
R7	5′-GCGAATTCTTAGGTTGTCAGGCAATA-3′	1677–1673

[a] Underlined nucleotides indicate sequences complementary to the genome of HCV.
[b] Corresponding amino acids in the HCV genome (13).

antibody (α-NS4B), anti-NS5A antibody (α-NS5A), anti-NS5B antibody (α-NS5B), and anti-DHFR antibody (α-DHFR) (10, 11, 15). α-NS4A and α-NS5A were generous gifts from A. Takamizawa (Osaka University, Osaka, Japan).

Pulse-chase analysis. After being incubated for 1 day in a 35-mm-diameter dish, COS-1 cells transfected with the series of pCMV-derived plasmids were used for pulse-chase analysis. Cells were incubated in 0.5 ml of methionine-free Eagle's minimum essential medium (Flow Laboratories) with 5% dialyzed fetal calf serum for 1 h, and then they were labeled for 15 min in the same medium supplemented with 400 μCi of [^{35}S]methionine (ICN) per ml. After pulse-labeling, the cells were either lysed immediately in 100 μl of Laemmli's sample buffer without dye or lysed following a chase for various times in Dulbecco's modified Eagle's medium containing methionine and cysteine (75 μg/ml each).

Immunoprecipitation of metabolically labeled cell extract with [^{35}S]methionine. Cell lysates metabolically labeled with [^{35}S]methionine were boiled and diluted 10-fold with extraction buffer (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). One milliliter of the diluted lysates was preadsorbed with 50 μl of protein G-Sepharose suspension (Pharmacia) and incubated with α-NS3, α-NS4A, α-NS4B, α-NS5A, and α-NS5B for 1 h. The immunocomplex was recovered by the addition of 30 μl of protein G-Sepharose and was washed three times with 50 mM Tris hydrochloride (pH 7.4)–500 mM NaCl–5 mM EDTA–1% Nonidet P-40–5% sucrose and once with 10 mM Tris hydrochloride (pH 7.4)–50 mM NaCl–1 mM EDTA. The immunoprecipitates were analyzed by SDS–8% PAGE or tricine–SDS–16% PAGE (18). The gels were dried and exposed to imaging plates (Fuji Photo Film Co., Ltd).

Subcellular localization of viral proteins. pCMV-derived plasmids were transfected into COS-1 cells as described above. After being incubated for 1 day in a 35-mm-diameter dish, the cells were incubated in 1 ml of methionine-free Eagle's minimum essential medium with 5% fetal calf serum supplemented with 200 μCi of [^{35}S]methionine per ml for 4 h. Cells were collected with a rubber scraper and homogenized in 200 μl of MSB medium (20 mM Tris hydrochloride [pH 8], 25 mM NaCl, 0.5 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). The homogenate was overlaid on 60% (lower layer) −40% (upper layer) sucrose solutions in MSB medium and centrifuged (200,000 × *g*, 60 min). After centrifugation, the portion on the 40% sucrose layer was defined as the cytosol fraction and the portion on the 60% sucrose layer was defined as the membrane fraction. Both fractions were diluted 10-fold with extraction buffer and immunoprecipitated as described above.

Nucleotide sequence accession number. The sequence of the cDNA of pCMV/N729-3010 has been deposited in the DDBJ, EMBL, and GenBank DNA databases under accession number D16435.

RESULTS

Effects of coproduced NS4A on the expression of HCV polyproteins encoded in pCMV/NSΔ4A. To understand the function of NS4A in HCV nonstructural polyprotein processing, pCMV/NSΔ4A, which encodes the entire putative HCV nonstructural polyprotein except the NS4A region, was expressed in COS-1 cells, and the protein products produced from this mutated polypeptide were analyzed with region-specific antisera. By comparing the products produced from NSΔ4A with those of the nonstructural region encoded in pCMV/N729-3010 (Fig. 1A), which produces all of the HCV nonstructural proteins, the function of NS4A in HCV nonstructural polyprotein processing was analyzed. The production of NS3, NS4A, NS4B, NS5A, and NS5B from N729-3010 (abbreviated as NS in Fig. 2) was detected by α-NS3, α-NS4A, α-NS4B, α-NS5A, and α-NS5B, respectively (Fig. 2, lanes 2). On the other hand, protein production from the NSΔ4A polypeptide was different from that from N729-3010 (Fig. 2A through D, lanes 3). The level of NS3 production was quite low

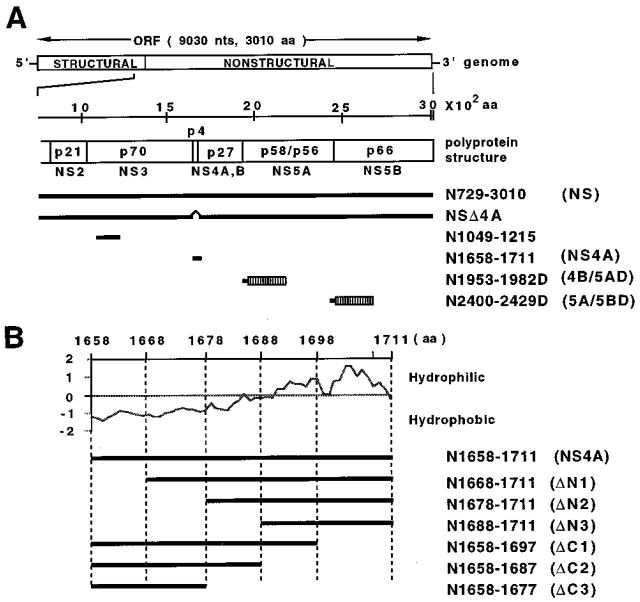

FIG. 1. Schematic representations of HCV polyprotein fragments produced by the expression constructs. (A) The genomic and polyprotein structures of the nonstructural region from NS2 to NS5B are shown enlarged below the HCV open reading frame (ORF). The scale indicates the amino acid position. The regions of polypeptides in the HCV precursor polyprotein are shown by thick bars. The designations of HCV polypeptide regions synthesized in COS-1 cells are shown on the right. Numbers indicate amino acid positions from the N terminus to the C terminus of the HCV precursor polyprotein. Abbreviations for the HCV polypeptides are indicated in parentheses. Hatched boxes indicate *E. coli* DHFR fused in frame at the C-terminal end of the HCV polypeptide (abbreviated as D in the peptide designations). nts, nucleotides. (B) HCV NS4A hydrophobicity profile as estimated by using the DNASIS program with a search length of 7 aa (Hitachi Software Engineering Co., Ltd.). A series of peptides with deletions from the N and C termini of NS4A is shown below the hydrophobicity profile.

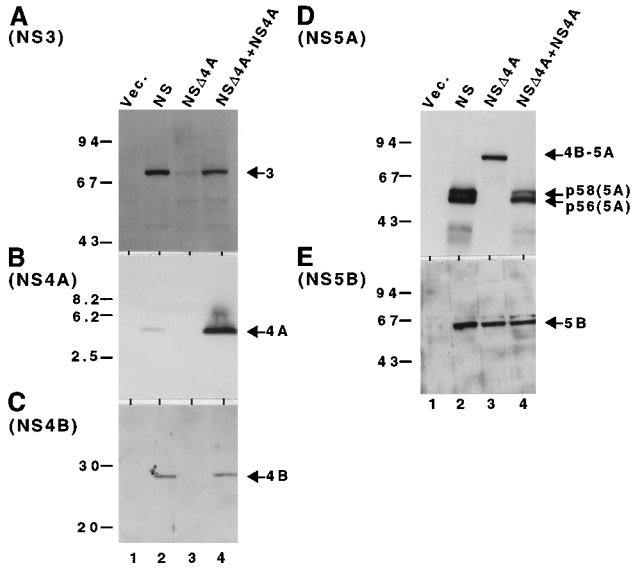

FIG. 2. Detection of processed products of NSΔ4A and the effect of NS4A on NSΔ4A processing. Lysates of COS-1 cells transfected with pKS(+)/CMV (10 μg), an original vector plasmid (Vec.) without an HCV insert (lanes 1); pCMV/N729-3010 (5 μg) plus pKS(+)/CMV (5 μg) (lanes 2); pCMV/NSΔ4A (5 μg) plus pKS(+)/CMV (5 μg) (lanes 3); or pCMV/NSΔ4A (5 μg) plus pCMV/N1658-1711 (5 μg) (lanes 4) were separated by SDS–8% PAGE (A, D, and E), SDS–10% PAGE (C), or tricine–SDS–16% PAGE (B) and analyzed by immunoblotting with α-NS3 (A), α-NS4A (B), α-NS4B (C), α-NS5A (D), or α-NS5B (E). Molecular mass markers (in kilodaltons) are shown on the left. The positions of processing products NS3, NS4A, NS4B, NS5A, and NS5B and the precursor polyprotein NS4B-NS5A are indicated on the right with arrows. NS, N729-3010.

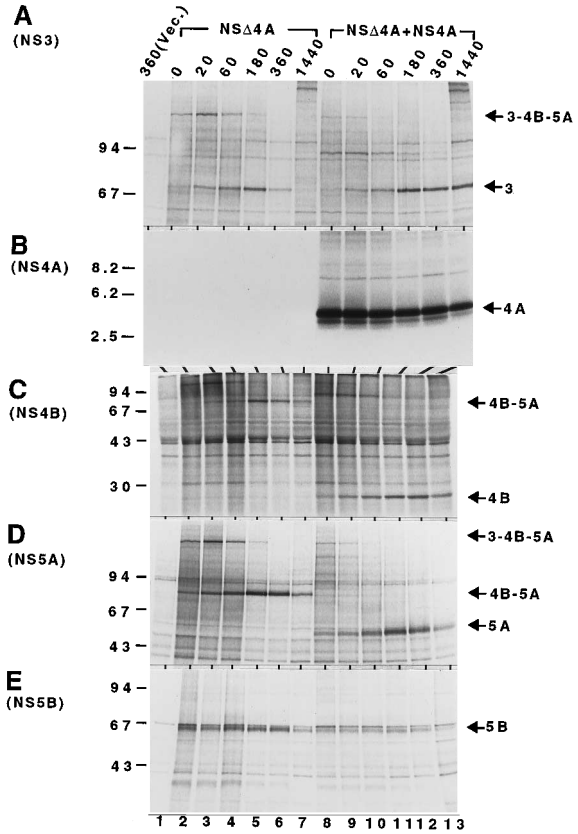

FIG. 3. Pulse-chase analysis of viral precursor polyprotein processing. Cells were transfected with pCMV/NSΔ4A (5 μg) plus pKS(+)/CMV (5 μg) or pCMV/NSΔ4A (5 μg) plus pCMV/1658-1711 (5 μg) and were pulse-labeled for 15 min and then chased as indicated. The lysate at each time point was immunoprecipitated with α-NS3 (A), α-NS4A (B), α-NS4B (C), α-NS5A (D), or α-NS5B (E). Immunoprecipitation was performed with lysates transfected with pKS(+)/CMV (lanes 1), pCMV/NSΔ4A (lanes 2 through 7), or pCMV/NSΔ4A plus pCMV/N1658-1711 (lanes 8 through 13). Gel conditions were as follows: SDS–8% PAGE for panels A, D, and E; SDS–10% PAGE for panel C; and tricine–SDS–16% PAGE for panel B. Molecular mass markers (in kilodaltons) are shown on the left. The positions of processing products NS3, NS4A, NS4B, NS5A, and NS5B and precursor polyproteins are shown on the right with arrows. Vec., vector.

in NSΔ4A. No NS4B or NS5A could be detected by α-NS4B or α-NS5A, respectively, while an 85-kDa polyprotein (p85) was detected by α-NS5A. Since the size of this polypeptide, 85 kDa, was almost identical to the sum of the molecular masses of NS4B and NS5A, this polypeptide was likely to be a processing intermediate composed of these two viral proteins. The failure of α-NS4B to detect p85 was possibly due to the low efficiency of blotting of the high-molecular-weight protein and the low titer of this antibody in this experiment. However, p85 was detected by α-NS4B in the pulse-chase experiment as described below. The production of NS5B as detected by α-NS5B indicates that cleavage at the 5A/5B site normally occurs in this mutant polypeptide. By coproduction with NS4A, the production of NS3, NS4B, and NS5A from NSΔ4A was restored (Fig. 2A, C, and D, lanes 4). The restoration of NS3 in the presence of NS4A seemed not to be due to efficient cleavage at the artificial NS3-NS4B site. Cleavage at this site occurs efficiently in the absence of NS4A, because the intermediate product, p85(NS4B-NS5A), accumulates in the absence of NS4A (Fig. 2D, lane 3). Instead, the restoration of NS3 by coproduction with NS4A seemed to be caused by the stabilization of NS3. The production of p58(NS5A) relative to p56(NS5A) generated by coproduction of NSΔ4A and NS4A was reduced compared with that for N729-3010 (Fig. 2D, lanes 2 and 4).

NS4A is necessary for the stabilization of NS3. Since cleavage at NS2/3 is not affected by viral nonstructural proteins encoded in the region downstream of NS3 (11), and the viral proteins p85(NS4B-NS5A) and NS5B, encoded in the region downstream of NS3, are produced from NSΔ4A, the greatly reduced production of NS3 from this mutated polypeptide was thought to be due to the instability of NS3 in cells producing the mutated polypeptide. To clarify this possibility, pulse-chase analysis using a transient expression system in COS-1 cells was conducted. Plasmid pCMV/NSΔ4A with or without pCMV/N1658-1711, which encodes NS4A, was used for transfection. Transfectants were pulse-labeled for 15 min with [^{35}S]methionine and chased for various times after the addition of excess amounts of unlabeled methionine. The cell lysates were analyzed at chase time points at 0, 20, 60, 180, 360, and 1,440 (1 day) min for the production of viral proteins by immunoprecipitation with α-NS3, α-NS4A, α-NS4B, α-NS5A, and α-NS5B.

Previously, we showed that the processing of NS3 from the precursor polyprotein of N729-3010 was almost completed within the 15-min pulse period and that NS3 was stably present for more than 1 day of the chase period (21). Contrary to this observation, the levels of NS3 in NSΔ4A-producing cell lysates were low at the start of the chase time, while a protein of approximately 150 kDa, immunoprecipitated by α-NS3 and likely to be the processing intermediate of NS3-NS4B-NS5A, was detected in significant amounts until 60 min of chase (Fig. 3A, lanes 2 through 5). The amount of NS3 reached a maximum at a chase time of 180 min and then started to decline.

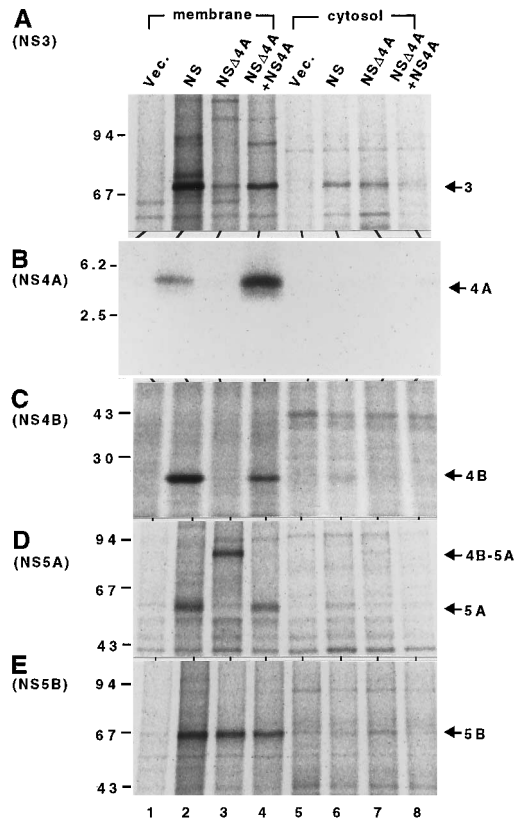

FIG. 4. Localization of processing products of NSΔ4A and the effect of NS4A on their subcellular localization. Lysates of COS-1 cells transfected with pKS(+)/CMV (10 μg) (lanes 1 and 5), pCMV/N729-3010 (5 μg) plus pKS(+)/CMV (5 μg) (lanes 2 and 6), pCMV/NSΔ4A (5 μg) plus pKS(+)/CMV (5 μg) (lanes 3 and 7), or pCMV/NSΔ4A (5 μg) plus pCMV/N1658-1711 (5 μg) (lanes 4 and 8) were separated into membrane (lanes 1 through 4) and cytosol (lanes 5 through 8) fractions as indicated in the text; fractionated by SDS–8% PAGE (A, D, and E), SDS–10% PAGE (C), or tricine–SDS–16% PAGE (B) after immunoprecipitation with α-NS3 (A), α-NS4A (B), α-NS4B (C), α-NS5A (D), or α-NS5B (E). Vec., vector. NS, N729-3010.

After 1 day (1,440 min) of chase, no detectable NS3 was present, suggesting that NS3 does not exist stably in the cell without NS4A. On the other hand, when NS4A was coproduced with NSΔ4A, the NS3 level increased until 180 min of chase, and the relative amount of NS3 remained almost constant thereafter, indicating that NS3 was stable in the cell (Fig. 3A, lanes 11 through 13). Coproduced NS4A was also stably present in the cell (Fig. 3B, lanes 8 through 13). A precursor polyprotein with a molecular mass of 85 kDa was detected by both α-NS4B and α-NS5A antibodies in lysates transfected with pCMV/NSΔ4A, but NS4B and NS5A were not detected throughout the chase period (Fig. 3C and D, lanes 2 through 7). On the other hand, NS4B and NS5A were produced when NS4A was coproduced with NSΔ4A (Fig. 3C, lanes 8 through 13, and 4D, lane 8). The production of NS5B from NSΔ4A was seen at the start of the chase, and the level of the protein was not affected by coproduction of NS4A (Fig. 3E, lanes 2 through 13).

Subcellular localization of HCV proteins. It is reported that NS4A is likely to associate with NS3 and that it is responsible for the membrane association of NS3 (12). Therefore, the prompt degradation of NS3 in the absence of NS4A was likely due to the loss of association of NS3 with the membrane. Association with the membrane may protect against proteolytic attack. To investigate this possibility, the role of NS4A in the localization of NS3 was examined (Fig. 4).

NS3, produced from N729-3010, was found in both the cytosol and membrane fractions (Fig. 4A, lanes 2 and 6). However, the amount of NS3 present in the membrane fraction was larger than that in the cytosol fraction. On the other hand, a larger amount of NS3 produced from NSΔ4A was detected in the cytosol fraction (Fig. 4A, lanes 3 and 7). A drastic change in the subcellular localization of NS3 was found when NS4A was coproduced with NSΔ4A. The majority of the NS3 was found in the membrane fraction (Fig. 4A, lanes 4 and 8). This result indicates that NS4A, which is known to associate with membrane, affects the localization of NS3, possibly through mutual interaction.

Besides NS3, the majorities of NS4A, NS4B, NS5A, NS5B, and related precursor polyproteins were detected in the membrane fraction both with and without NS4A (Fig. 4B through E).

Role of NS4A in HCV nonstructural protein processing. To investigate the role of NS4A in HCV nonstructural protein processing, a viral serine proteinase (Cpro-2)-dependent *trans* cleavage assay was performed. The function of NS4A in the processing of HCV nonstructural proteins was assayed by cleavage of DHFR fusion peptides containing 30 amino acid residues around the Cpro-2 cleavage site with a protein, N1049-1215, which has a Cpro-2 activity. The 30 aa used for this assay consisted of 20 aa located upstream of each cleavage site and 10 aa located downstream of each cleavage site. *E. coli* DHFR was fused in frame at the C-terminal end of each 30-aa segment to facilitate the detection of the C-terminal cleavage product by α-DHFR (11). Cleavage was detected in the substrate proteins of 4B/5AD and 5A/5BD. Both enzyme and NS4A proteins were required for the cleavage at 4B/5A (Fig. 5, lanes 1 through 3). The fact that NS4A was necessary for cleavage at the 4B/5A site implies that 30 aa around the 4B/5A site are sufficient for cleavage by N1049-1215 plus NS4A. On the other hand, approximately half of the 5A/5BD was processed by enzyme protein only, while coproduction of NS4A led to an increase in cleavage efficiency (Fig. 5, lanes 4 through 6). The faint band seen in the same position as the cleaved product in the absence of Cpro-2 may be a degraded product (Fig. 5, lane 4).

Stoichiometrical assay of NS4A function. To investigate the stoichiometrical relationship between NS4A and the enzyme-substrate protein of NSΔ4A, we examined the cleavage efficiency at the 4B/5A site by using different amounts of pCMV/NSΔ4A and pCMV/N1658-1711 plasmids for transfection. The efficiencies of 4B/5A cleavage were evaluated by detecting cleaved NS5A products with α-NS5A. As shown in Fig. 6, the production of NS5A was reduced to an extent corresponding to the decrease in the amount of NS4A-encoding plasmid. However, production of p56(NS5A) was detected by α-NS5A even when the amount of NS4A-encoding plasmid was far less than that of the NSΔ4A-encoding plasmid. A proportional relationship between the production of protein and the amount of the plasmid was found if the amount of plasmid used was less than 5 μg per well in this assay system (data not shown). Therefore, it was obvious that the presence of the smallest amount of NS4A relative to NS3 was sufficient to cleave at the 4B/5A site of NSΔ4A. For instance, even if the ratio of pCMV/NSΔ4A to pCMV/N1658-1711 was 99 μg to 1 μg (i.e., the molar ratio of nonstructural polyprotein to NS4A was 38:1), more than half of the substrate protein NSΔ4A was cleaved at the 4B/5A site to produce p56(NS5A) (Fig. 6, lane 8).

The production of p58(NS5A) was strongly influenced by the amount of NS4A-encoding plasmid. When excess amounts

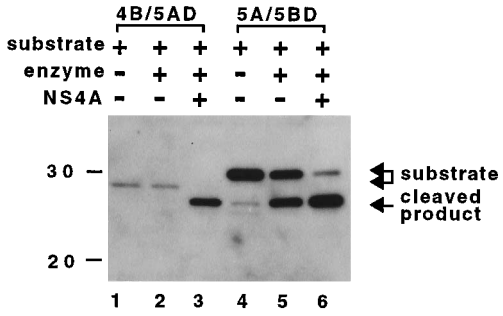

FIG. 5. Role of NS4A in HCV nonstructural protein processing. Lysates of COS-1 cells transfected with the plasmids indicated above the gel were fractionated by SDS–10% PAGE and analyzed by immunoblotting with α-DHFR. N1049-1215, a serine proteinase encoded by pCMV/N1049-1215, was used as the enzyme protein. Substrate proteins were 4B/5AD (lanes 1 through 3) and 5A/5BD (lanes 4 through 6). The amounts of the plasmids used were as follows: substrate-encoded plasmid (3.3 μg) plus pKS(+)/CMV (6.7 μg) (lanes 1 and 4); substrate-encoded plasmid (3.3 μg) plus enzyme-encoded plasmid (3.3 μg) plus pKS(+)/CMV (3.3 μg) (lanes 2 and 5); and substrate-, enzyme-, and NS4A-encoded plasmids (3.3 μg each) (lanes 3 and 6). Molecular mass markers (in kilodaltons) are shown on the left. The positions of the substrate protein and cleaved products are indicated on the right with arrows.

of NS4A relative to NSΔ4A were expressed in the cell, the relative production of p58(NS5A) and p56(NS5A) was almost the same as that in lysates of N729-3010-producing cells (Fig. 6, lanes 1 through 3). On the other hand, when the smaller amount of NS4A relative to NSΔ4A was expressed in the cell, the relative production of p58(NS5A) was reduced (Fig. 6, lanes 5 through 8). When 0.2 or 0.1 μg of plasmid encoding NS4A was used for transfection, no p58(NS5A) production was detected, while the uncleaved p85 product was detected (Fig. 6, lanes 7 and 8). These results suggest that NS4A also controls the production of p58(NS5A). Equal or excess amounts of NS4A relative to the enzyme-substrate protein (NSΔ4A) were required for the production of p58(NS5A).

Deletion mapping of NS4A. As shown in Fig. 1B, NS4A consists of two distinct parts, an N-terminal hydrophobic half and a C-terminal hydrophilic half. The roles of these two regions in NS4A functioning are not known. An assay of NS4A-dependent cleavage at the 4B/5A site was used to determine the region of the NS4A protein essential for this function. A series of deletion mutants (Fig. 1B) derived from N1658-1711

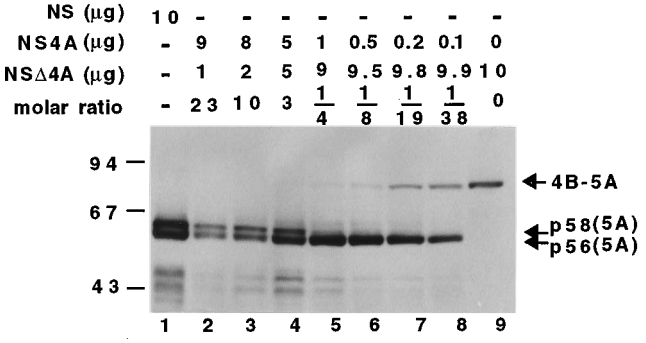

FIG. 6. Stoichiometrical assay of NS4A function in 4B/5A cleavage. Lysates of COS-1 cells transfected with 10 μg of a plasmid encoding NS (lane 1) or with different relative amounts of plasmids encoding NSΔ4A and NS4A (lanes 2 through 9) were fractionated by SDS–8% PAGE. The amounts of plasmids used and the molar ratios of pCMV/N1658-1711 to pCMV/NSΔ4A are indicated at the top. Molecular mass markers (in kilodaltons) are shown on the left. The positions of the processing products are indicated on the right with arrows.

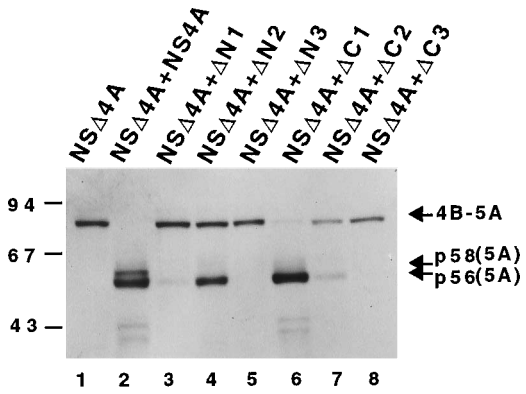

FIG. 7. Effects of NS4A products containing deletions on cleavage at the 4B/5A site. Lysates of COS-1 cells transfected with a plasmid encoding NSΔ4A (5 μg) plus pKS(+)/CMV (5 μg) (lane 1) or a plasmid encoding NS4A or one of its deletion mutants (5 μg each) (lanes 2 through 8) were fractionated by SDS–8% PAGE and then analyzed by immunoblotting with α-NS5A.

(NS4A) was used for the assay. The activity of each deletion mutant was determined by detecting NS5A production from NSΔ4A. When ΔN1, ΔN2, ΔC1, and ΔC2 were coproduced with NSΔ4A, different levels of p56(NS5A) were detected by α-NS5A (Fig. 7, lanes 3, 4, 6, and 7). The production of NS5A was greatly reduced when ΔN1 or ΔC2 was used as the source of NS4A. However, there was no NS5A production when ΔN3 or ΔC3 was coproduced. These data indicate that the C-terminal part of NS4A downstream of aa 1688 and the N-terminal part upstream of aa 1677 are not essential for NS4A activity, although a significant effect of these regions on cleavage at the 4B/5A site was observed. The minimum domain for NS4A activity appears to be from aa 1678 to aa 1687 of the HCV precursor polyprotein, although whether or not the expected minimum domain of N1678-1687 itself is functional remains to be determined.

DISCUSSION

The transient expression system in COS-1 cells and pulse-chase analysis showed the production of NS3 and NS5B from NSΔ4A, indicating that cleavage at the NS2/3 and 5A/5B sites proceeds without the presence of NS4A. However, the rate of NS3 production in this construct was delayed compared with that in N729-3010 (21). During 180 min of chase, a putative precursor protein was detected by α-NS3, suggesting that processing to NS3 was not completed by the end of the 180-min chase. Since NS4A was excluded from the mutated polypeptide NSΔ4A, NS4B was followed directly by NS3. Therefore, the amino acid sequence around the C-terminal end of NS3 was changed from DLEVVT/STW to DLEVVT/ASH (/ indicates the cleavage site). It is possible that the delayed processing of NS3 in NSΔ4A compared with the result for N729-3010 was caused by the artificial amino acid sequence between NS3 and NS4B. However, this change of amino acid sequence at the cleavage site seems not to affect the further processing of nonstructural precursor polyprotein. On the other hand, the production of NS5B in lysates transfected with pCMV/NSΔ4A was detected at the end of the pulse period, and no precursor protein reactive with α-NS5B was detected. Therefore, efficient cleavage proceeds at the 5A/5B site of the NSΔ4A polyprotein in the absence of NS4A.

The amount of NS3 produced from NSΔ4A in the absence of NS4A reached a maximum after 180 min of chase, and then

it decreased gradually. In contrast, the level of NS3 remained almost unchanged from the level it reached after 180 min of chase in the presence of NS4A. Furthermore, a smaller amount of NS3 production was observed in cells producing only the NSΔ4A polyprotein than in cells coproducing NSΔ4A polyprotein and NS4A (Fig. 3A, lanes 3 and 4). Since the 85-kDa NS4B-NS5A precursor protein was clearly seen in this experiment (Fig. 2D, lane 3), processing of the C terminus of NS3 must occur properly. These observations suggest that NS4A plays an important role in the stabilization of NS3.

An analysis of the subcellular localization of the nonstructural proteins revealed that without the coexistence of NS4A, more than 50% of the NS3 produced from NSΔ4A was localized in the cytosol fraction, while when NS3 was coproduced with NS4A, most of the NS3 was found in the membrane fraction. Previously we showed that a possible association between NS4A and NS3 is important for the membrane anchoring of NS3 (12). In view of the previous data and of the evidence presented here, one aspect of NS4A functioning may be to anchor NS3 to the membrane of the endoplasmic reticulum.

Since NS5B was produced efficiently from NSΔ4A, it is believed that NS4A is not essential for cleavage at the 5A/5B site as reported by other groups (3, 7). However, it is noteworthy that the cleavage at the 5A/5B site of the 5A/5BD protein was enhanced when NS4A was present (Fig. 5, lanes 5 and 6). In this assay system, the serine proteinase domain was supplied in *trans* to the substrate, which contained only 30 amino acid residues surrounding the cleavage site; thus, the efficiency of cleavage might have been lowered. The molecular mechanism for the enhancement of 5A/5B cleavage by Cpro-2 with NS4A is not clear. However, stabilization of Cpro-2 and membrane association of Cpro-2 caused by NS4A, which may facilitate close association with substrates, may be considered to enhance the cleavage function.

The fact that the presence of NS4A is indispensable for processing at the 4B/5A site was confirmed in this study as it has been by others (3, 7). The possible function of NS4A in stabilizing NS3 may not be sufficient for this cleavage because NS3 could be detected in the absence of NS4A by the time that cleavage at the 4B/5A site was completed in the presence of NS4A (Fig. 3A and D). Thus, it is likely that an additional function of NS4A, which cooperates with the function of NS3, involves cleavage at the 4B/5A site. For this function, 10 amino acid residues harboring the central region of NS4A (aa 1678 to 1687) were shown to be essential, although a great reduction in activity by this limited domain was evident (Fig. 7). The serine proteinase domain, aa 1049 to 1215, was sufficient to cleave at the 4B/5A site of a substrate containing only 30 amino acid residues surrounding the 4B/5A cleavage site in the presence of NS4A (Fig. 5). This result is in contrast to the results of Bartenschlager and coworkers (3), in which the N-terminal truncation of NS3 abolished NS4A-dependent 4B/5A cleavage. The domain of Cpro-2 (N1049-1215) used in the present study contains a further deletion of 15 aa from the N terminus of their construct. The reason for the conflicting results is not known; however, they may have been caused by the use of HCV clones with different sequences or by differences in the assay systems.

Proteins with different molecular weights, p56(NS5A) and p58(NS5A), were produced from NS5A. The biochemical nature of these proteins remains to be clarified. The production of p58(NS5A) from mutated HCV nonstructural polyproteins in which amino acids at the P1 and P1′ positions in the 3/4A or 4A/4B sites are replaced with asparagine is drastically reduced, leading to impaired cleavage at these sites (21). These results imply that only the authentic product of NS4A allows the production of p58(NS5A). This is in agreement with our observation that all of the truncated forms of NS4A fail to produce p58(NS5A).

Results from the stoichiometrical assay also reveal that the production of p58(NS5A) is strongly controlled by the level of NS4A. For the production of p58(NS5A), an equal or greater amount of NS4A relative to NSΔ4A is required. However, a lesser amount of NS4A is needed for cleavage at 4B/5A site. The mechanism for control of the production of p58(NS5A) by NS4A remains to be clarified. The essential function of NS4A in the production p58(NS5A) differs from that in the cleavage at the 4B/5A site.

In this paper we describe the versatile functions of NS4A of HCV. The function of NS4A seems to differ from that of the NS2B product of flaviviruses, which is required for NS3-dependent proteolytic cleavage of the flavivirus precursor polyprotein (1, 4, 8, 14, 17). The complicated function of NS4A seems to be involved not only in the strict regulation of the proteolytic processing of the precursor polyprotein but also in the function of the processed protein, a function that may be crucial for viral proliferation.

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ADDENDUM IN PROOF

We observed that NS5A products are phosphorylated and the larger product is the hyperphosphorylated form of the smaller product. Production of the hyperphosphorylated form depends on the presence of NS4A (T. Kaneko, Y. Tanji, S. Satoh, M. Hijikata, S. Asabe, K. Kimura, and K. Shimotohno, Biochem. Biophys. Res. Commun. **205:**320–326, 1994).

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