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Molecular Determinants for Subcellular Localization of Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein is a putative nucleocapsid protein with a number of regulatory functions. In tissue culture cells, HCV core protein is mainly located at the endoplasmic reticulum as well as mitochondria and lipid droplets within the cytoplasm. However, it is also detected in the nucleus in some cells. To elucidate the mechanisms by which cellular trafficking of the protein is controlled, we performed subcellular fractionation experiments and used confocal microscopy to examine the distribution of heterologously expressed fusion proteins involving various deletions and point mutations of the HCV core combined with green fluorescent proteins. We demonstrated that a region spanning amino acids 112 to 152 can mediate association of the core protein not only with the ER but also with the mitochondrial outer membrane. This region contains an 18-amino-acid motif which is predicted to form an amphipathic α-helix structure. With regard to the nuclear targeting of the core protein, we identified a novel bipartite nuclear localization signal, which requires two out of three basic-residue clusters for efficient nuclear translocation, possibly by occupying binding sites on importin-α. Differences in the cellular trafficking of HCV core protein, achieved and maintained by multiple targeting functions as mentioned above, may in part regulate the diverse range of biological roles of the core protein.

Hepatitis C virus (HCV), the most important causative agent of posttransfusion and sporadic non-A, non-B hepatitis, is a positive-stranded RNA virus belonging to the family *Flaviviridae* (7). A precursor polyprotein of about 3,000 amino acids is encoded by a large open reading frame of the genome and undergoes cellular and viral protease-mediated posttranslational modification to produce a series of structural and nonstructural proteins (8, 13, 16).

HCV core protein, which is derived from the N terminus of the viral polyprotein, forms multimers and interacts physically with the viral RNA to constitute the nucleocapsid (28, 47, 50). Tissue transglutaminase is responsible for stabilizing the core protein by cross-linking it into a dimeric form (26). In addition, the core viral protein has properties which enable it to modulate a number of cellular processes, including transcription, inhibition or stimulation of apoptosis, and suppression of host immunity, as reviewed previously (21, 29, 51, 52). Several studies suggest that expression of the core protein affects mitochondrial function and lipid metabolism. The core protein increases the cellular production of reactive oxygen species with subsequent increases in lipid peroxidation (35, 39). The viral protein also colocalizes with human apolipoprotein AII, associates with lipid droplets, and has the capacity to influence metabolic events involving lipid storage (2, 17, 30, 36, 44). In addition, the core protein reduces microsomal triglyceride transfer, leading to defects in very low density lipoprotein assembly and secretion (40). Furthermore, the HCV core protein has transforming potential in some cells under certain conditions (5, 42). Transgenic mice expressing this protein in the liver develop hepatic steatosis due to increased oxidative stress in the absence of inflammation, with subsequent development of hepatocellular carcinoma (34, 36). These results suggest that the HCV core protein might play a pivotal role in the pathogenesis of hepatitis C in addition to its role as a structural component of the viral capsid.

The amino acid sequence of the core protein is well conserved among different HCV isolates and genotypes compared to other HCV proteins. The N-terminal domain of the HCV core protein is highly basic, while its C terminus is hydrophobic. Although several core proteins of various sizes exist (17 to 23 kDa) (15, 23, 25, 49, 56), two processing events result in the predominant production of a 21-kDa core protein. Both of these events utilize the endoplasmic reticulum (ER). The first one is to be cleaved from downstream envelope protein E1 at position 191, where the C-terminal hydrophobic domain serves as a putative signal peptide sequence. Subsequently, the signal sequence of 13 or 18 residues is processed by signal peptide peptidase (19, 23, 56).

The HCV core protein is found primarily within the membranes of cytoplasmic organelles, but it is also found in the nucleus (23, 48, 56). Immunofluorescence studies show a punc-

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tate pattern, consistent with ER localization, as well as perinuclear localization (15, 24, 32, 46, 56). Some studies suggest direct effects of the core protein on mitochondrial function. In fact, the core protein localizes to the mitochondria (34, 39). The N-terminal domain of the core protein contains three stretches of arginine- and lysine-rich sequences. Translocation of the core protein to the nucleus, mediated by these basic-residue stretches which function as nuclear localization signals (NLSs), is observed (6, 48). In addition, Moriishi et al. demonstrated that the N-terminal region of the core protein is also essential for nuclear retention through its interaction with the proteasome activator PA28γ (33).

In this study, we found a region that is important for localization of the mature core protein to the ER and to the mitochondrial outer membrane. We also identified a novel bipartite NLS responsible for nuclear targeting of the core protein, presumably via an importin-dependent pathway.

MATERIALS AND METHODS

Plasmid construction. The construction of a plasmid expressing the full-length core protein of 191 amino acids, pCAGC191, was described previously (49). pGFP, a construct expressing green fluorescent protein (GFP) with a C-terminal Myc epitope tag sequences, was prepared as follows. pCMV/Myc/mito/GFP (Invitrogen Corp., Carlsbad, Calif.) was digested with PmlI, followed by treatment with the Klenow fragment of DNA polymerase I. The resultant linear fragment was ligated to a PstI linker (GCTGCAGC) and digested with PstI to remove the mitochondrial targeting signal sequence, followed by self-ligation. A series of HCV core-GFP fusion constructs were made by amplifying the core gene fragments with PCR with primers containing Flag epitope tag sequences (sense) and a PstI site (both). After digestion with PstI, the segments were inserted into the PstI site of pGFP. A series of GFP-core-E1 fusion constructs were made by amplifying core and E1 gene fragments with PCR with primers containing a NotI site. After digestion with NotI, the segments were inserted into the NotI site of pGFP.

pGEX-4T-1 (Amersham Bioscience Corp., Piscataway, N.J.) was used to express core protein fused with glutathione *S*-transferase (GST) in *Escherichia coli*. Core cDNA fragments encoding amino acids 1 to 71 were inserted into the EcoRI site of pGEX-4T-1. Alanine substitutions were introduced into the core protein by PCR mutagenesis with primers containing base alterations. The PCR products were then cloned into pCR2.1 (Invitrogen Corp.) and verified by DNA sequencing. Individual cDNAs were excised and inserted separately into pGFP or pGEX-4T-1. The primer sequences used in this study are available from the authors upon request.

Plasmid pRSET-hSRP1α (54), containing importin-α cDNA under the control of a T7 promoter, was kindly provided by Karsten Weis (University of California, Berkeley). A cDNA clone of importin-α possessing 14 residues (MYPYDVP DYGGGGS), derived in part from the hemagglutinin (HA) tag at the N terminus, was constructed by PCR. The resultant linear fragment was inserted under the control of a CAG promoter of pCAGGS and designated pCAG-HA-imp.

Cell culture and transfection. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Monolayers of 293T cells were transfected with plasmid DNA in the presence of Lipofectamine (Gibco-BRL, Life Technologies, Gaithersburg, Md.) according to the manufacturer's instructions.

Confocal immunofluorescence microscopy. Transfected cells were grown on glass coverslips. Two days after transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. Intracellular localization of HCV core-GFP fusion proteins was visualized in cells transfected with a variety of GFP fusion constructs.

In order to detect the HCV core protein by immunofluorescence, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 3 min at room temperature, followed by blocking with a nonfat milk solution (Block Ace; Snow Brand Milk Products Co., Sapporo, Japan). The cells were then incubated with anticore monoclonal antibody B2 (Anogen, Mississauga, Canada) for 60 min at room temperature, followed by incubation with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (IgG) (ICN Pharmaceuticals, Aurora, Ohio) for 45 min. To visualize mitochondria, MitoTracker Red CM-H_2XRos (Molecular Probes, Eugene, Oreg.) was added to the culture medium to a final concentration of 100 nM and incubated for 120 min at 37°C prior to fixation. To visualize the ER, goat anticalregulin antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) and rhodamine-conjugated rabbit anti-goat IgG (ICN Pharmaceuticals) were used as the first and second antibodies, respectively. To visualize HA-importin-α, mouse anti-HA antibody (Roche Molecular Biochemicals, Indianapolis, Ind.) and rhodamine-conjugated goat anti-mouse IgG (ICN Pharmaceuticals) were used as the first and second antibodies, respectively. All specimens were examined with an LSM510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Immunoelectron microscopy. Cells were transfected as described above. After 2 days, cells were fixed with 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS (pH 7.4). Free aldehyde groups were quenched with 50 mM NH_4Cl in PBS. The cell pellets were embedded at progressively lower temperatures (down to −35°C) in Lowicryl k4M according to an established protocol (43). Ultrathin sections were prepared and mounted on carbon-coated nickel grids. To perform electron microscopy, Lowicryl k4M ultrathin sections, mounted on grids, were floated on a droplet of PBS containing 1% bovine serum albumin, 0.1% Triton X-100, and 0.1% Tween 20 for 10 min, after which they were exposed to droplets of mouse anticore monoclonal antibody (Anogen) diluted in PBS for 45 min. Following this, they were rinsed twice for 5 min each in PBS and incubated with anti-mouse IgG-coated 10-nm immunogold particles (British Biocell, Cardiff, United Kingdom) for 45 min. After rinsing with PBS and distilled water, the grids and embedded sections were air dried and exposed to uranyl and lead acetate contrast agents.

Subcellular fractionation. All steps were performed at 4°C in the presence of a protease inhibitor cocktail called Complete (Roche Molecular Biochemicals). To isolate the ER fraction, transfected cells were washed with PBS, lysed in homogenization buffer A (50 mM Tris-HCl [pH 8.0], 1 mM β-mercaptoethanol, 1 mM EDTA, and 0.32 M sucrose), and then centrifuged at 5,000 × *g* for 10 min. The supernatant was then collected and centrifuged at 105,000 × *g* for 1 h. The pellet was disrupted in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and 10 mM sodium fluoride), after which it was centrifuged at 15,000 × *g* for 20 min. The resulting supernatant was used as the ER fraction.

To isolate the mitochondrial fraction, transfected cells were washed with PBS and homogenized in ice-cold homogenization buffer B (200 mM mannitol, 50 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl) at pH 7.4. The supernatant was then centrifuged at 1,000 × *g* for 10 min to remove large debris and nuclei. The resulting supernatant was then centrifuged at 20,000 × *g* for 20 min to obtain crude mitochondria. The crude mitochondria pellet was subfractionated in Nycodenz gradients for further purification of mitochondria. Nycodenz (Axis-Shield PoC AS, Oslo, Norway) solution at 50% (wt/vol) was prepared in buffer containing 5 mM Tris-HCl and 1 mM EDTA at pH 7.4. This stock solution was then diluted with buffer containing 0.25 M sucrose, 5 mM Tris-HCl, and 1 mM EDTA at pH 7.4 before use. The crude mitochondrial pellets was suspended in 4 ml of 25% Nycodenz solution and overlaid onto the following discontinuous Nycodenz gradients: 1 ml of 40%, 1 ml of 34%, and 2 ml of 30%. The samples were topped off with 2 ml of 23% Nycodenz solution after placement onto the discontinuous gradients. The tubes were then centrifuged at 52,000 × *g* for 90 min. The dense band seen after centrifugation at the 25 to 30% interface was recovered as the purified mitochondrial fraction.

To determine the submitochondrial localization pattern of the core protein, mitochondria were resuspended in SH buffer (0.6 M sorbitol and 20 mM HEPES-KOH [pH 7.2]) in the absence or presence of 30 μg of proteinase K per ml after purification by Nycodenz density gradient centrifugation. Samples were incubated for 30 min at 0°C, after which protease digestion was halted by the addition of *p*-aminophenyl methanesulfonyl fluoride hydrochloride (*p*-APMSF) (5 mM). Proteins lysed in sodium dodecyl sulfate (SDS) sample buffer were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted as described below.

Immunoblot analysis. The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Tokyo, Japan) after separation by SDS-PAGE. After blocking, the membranes were probed with monoclonal- or polyclonal-antibody against core protein (Anogen), prohibitin (Neo Markers, Fremont, Calif.), ribophorin I (Santa Cruz Biotechnology), translocase of the outer membrane (Tom) 20 (Santa Cruz Biotechnology), translocase of the inner membrane (Tim) 17 (Santa Cruz Biotechnology), or GFP (Santa Cruz Biotechnology). Immunoblots were developed as previously described (15).

GST pulldown assay. *Escherichia coli* BL21 cells were transformed with GST-core fusion plasmids and grown at 37°C. Expression of the fusion protein was induced by 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3 h. Bacteria were harvested, suspended in lysis buffer (1% Triton X-100 in PBS), and soni-

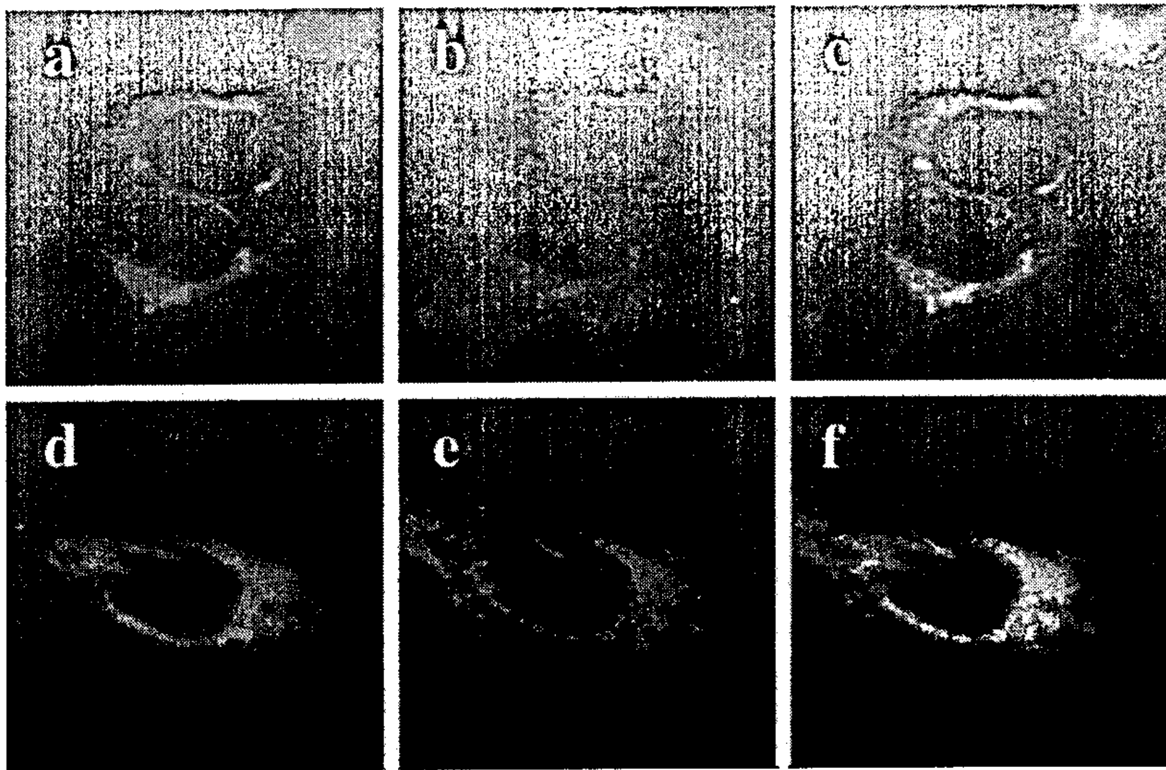

FIG. 1. Confocal analysis of double staining for HCV core protein and ER or mitochondria. 293T cells transfected with full-length HCV core expression plasmid, pCAGC191 were allowed to express the plasmid for 2 days. Transfected cells were fixed directly (a to c) or fixed after loading with Mitotracker (d to f). After permeabilization with Triton X-100, cells were subjected to immunofluorescence staining with a mouse anticore antibody. A goat anticalregulin antibody was used for ER staining. The green signals corresponding to the core were found with a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (a and d). The red signals corresponding to the ER were obtained with a rhodamine-conjugated rabbit anti-goat IgG secondary antibody (b). Mitochondria were stained with the mitochondrion-selective dye Mitotracker (e). Overlay resulted in yellow signals indicative of colocalization (c and f).

cated on ice. GST and GST fusion proteins were purified from bacterial lysates with glutathione-Sepharose beads (Amersham Bioscience Corp.). The beads were washed four times with lysis buffer. Approximately equal amounts of purified protein, as estimated by Coomassie brilliant blue staining, were used for the binding assays. For pulldown assays, in vitro transcription and translation of importin-α was done with pRSET-hSRP1α and the TNT-coupled reticulocyte lysate system (Promega Corp., Madison, Wis.) with T7 RNA polymerase. The reaction was carried out at 30°C for 4 h in the presence of [^{35}S]methionine/cysteine (ICN Pharmaceuticals). The translation product was then incubated with glutathione-Sepharose beads bound to GST fusion proteins in 1 ml of binding buffer (40 mM HEPES [pH 7.5], 100 mM KCl, 0.1% NP-40, and 20 mM 2-mercaptoethanol) at 4°C for 1 h. The beads were washed four times with binding buffer, and the pulldown complexes were separated by SDS-PAGE on 15% polyacrylamide gels. The gels were then fixed, dried, and analyzed with autoradiography.

RESULTS

Subcellular localization of HCV core protein. To assess the subcellular localization of HCV core protein, we first analyzed cells transfected with a full-length core-expressing construct by confocal microscopy. In accordance with previous observations (2, 15, 32, 45, 56), a granular cytoplasmic staining pattern of the core protein was observed in 293T (Fig. 1) and human hepatoblastoma HepG2 (data not shown) cells. Dual staining of transfected cells with antibody against the ER protein calregulin along with anticore antibody confirmed the ER localization of the core protein (Fig. 1a, b, and c show the core,

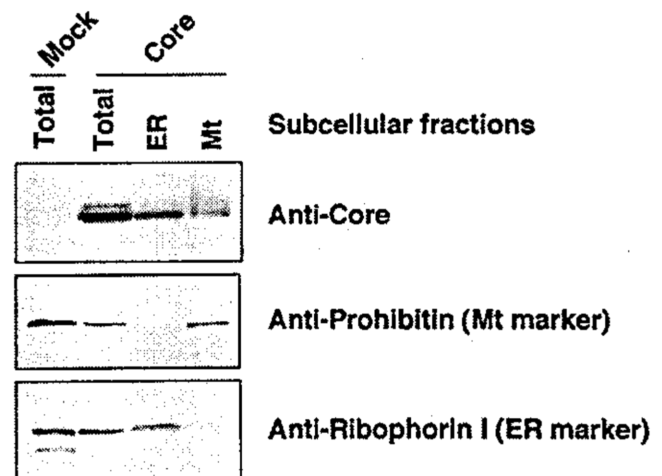

FIG. 2. Subcellular distribution of HCV core protein analyzed by immunoblotting. ER and mitochondrial (Mt) fractions were isolated from 293T cells expressing the full-length core protein (Core) or nontransfected cells (Mock) 2 days after transfection. Equal amounts of protein from each fraction as well as whole cell lysates (Total) were subjected to immunoblotting with a monoclonal antibody against either HCV core, prohibitin, or ribophorin I.

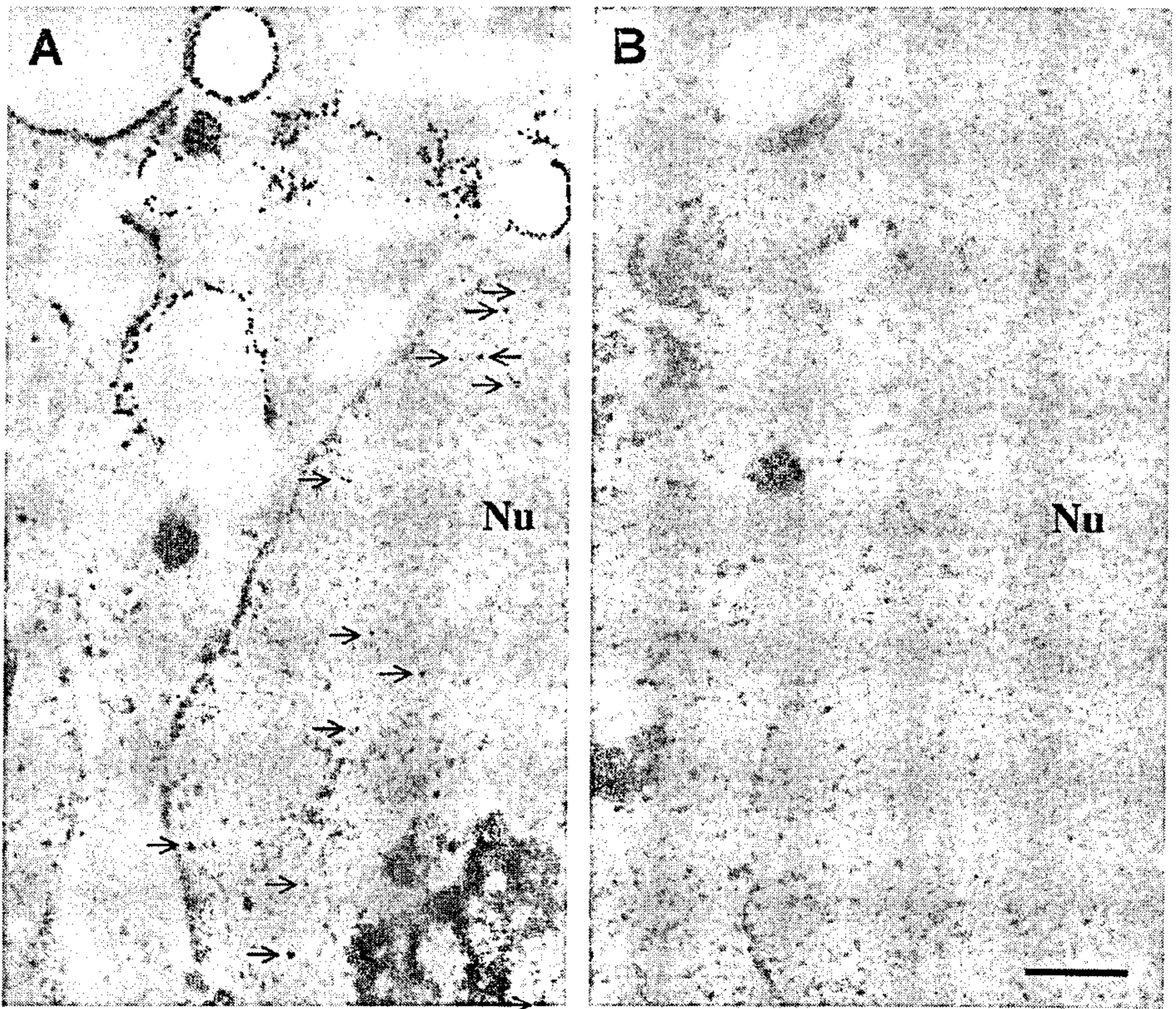

FIG. 3. Immunoelectron microscopy of HCV core protein. 293T cells expressing the full-length core protein (A) and nonexpressing cells (B) fixed 2 days after transfection. Immunoelectron microscopic analysis was performed with a mouse anticore antibody and a secondary anti-mouse IgG conjugated with gold particles. The arrows indicate the core protein localized in the nucleus (Nu). Bar, 500 nm.

calregulin, and a merged image, respectively). The pattern of subcellular localization of the core protein (Fig. 1d) was compared to the distribution of mitochondria, as revealed by MitoTracker staining (Fig. 1e). Although distribution of the core protein was not completely identical with that of the mitochondrion-selective dye, overlapping staining was observed, particularly in the perinuclear region (Fig. 1f).

Intracellular localization of the core protein was further examined in 293T cells by subcellular fractionation and Western blotting. The core protein was present in both the ER and mitochondrial fractions (Fig. 2), while it was not detected in the cytosol fraction (data not shown). The purity of the ER and mitochondrial fractions was confirmed with antibodies against ribophorin I as an ER marker and prohibitin as a mitochondrial marker.

It is generally difficult to identify the nuclear distribution of proteins of interest due to contamination of the nuclear preparation with unbroken, intact cells. Thus, to investigate whether the core protein localizes to the nucleus, we examined transfected cells by immunoelectron microscopy. Although gold particles were primarily observed within cytoplasmic membranes, perhaps highlighting the ER, immunoreactivity to anticore antibody was also observed in the nucleus (Fig. 3A, arrows). In contrast, no antibody labeling was observed in cells transfected with an empty vector (Fig. 3B).

Thus, HCV core protein predominates in the cytoplasm in a membrane-associated form(s) with ER and mitochondria, but nuclear localization is also observed.

Regions responsible for directing core protein to the ER and mitochondria. Given the tendency of the core protein to lo-

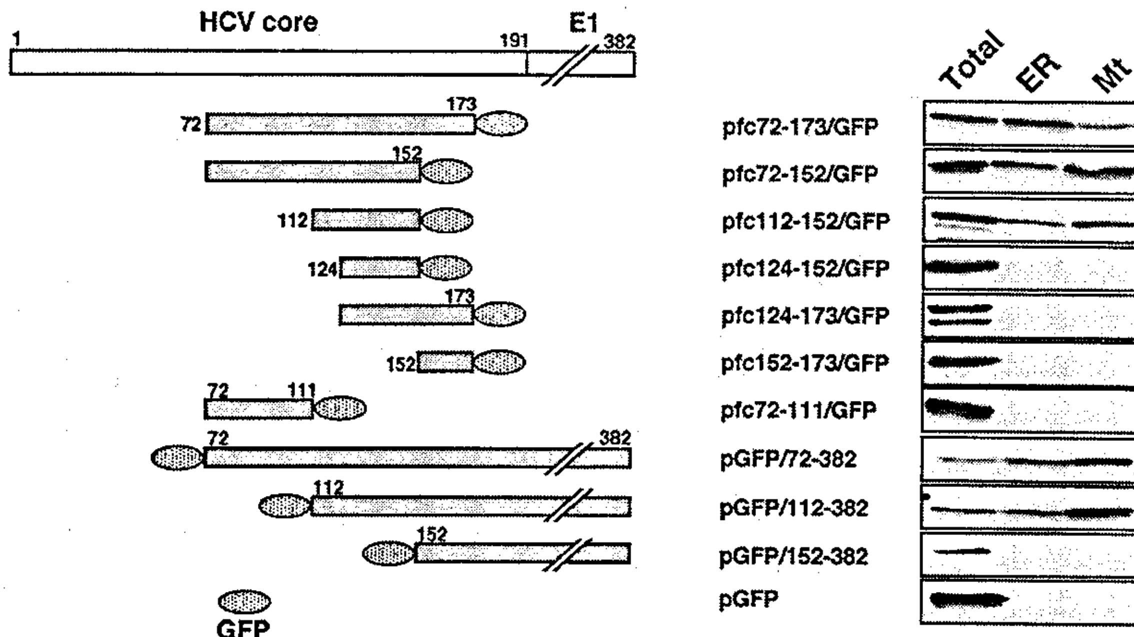

FIG. 4. Identification of segments that mediate association with ER and mitochondria in the core protein. Schematic diagram (left) and nomenclature (middle) of the core-GFP fusions are shown. Gray bars, expressed core and E1 regions. Subcellular distribution of fusion proteins is indicated on the right. ER and mitochondrial (Mt) fractions as well as whole-cell lysates (Total) were subjected to immunoblotting with an anti-GFP antibody.

calize to the ER and to mitochondria, we next investigated whether specific sequences might be responsible for transporting the core protein to these organelles. Fusion proteins between different regions of the core protein and GFP were developed, with specific emphasis on the region downstream of amino acid 72 because this region contains clusters of hydrophobic amino acids and the N-terminal 71 residues of the core are known to play a role in nuclear targeting (6, 48).

Western blotting of subcellular fractions with anti-GFP antibody revealed the localization of a core (72–173)-GFP fusion protein to the ER and to mitochondria (Fig. 4). Fusion proteins containing GFP and core proteins with N- or C-terminal deletions (72-152-GFP and 112-152-GFP) were likewise identified within the ER and mitochondrial fractions. In contrast, the ER and mitochondrial fractions did not contain GFP fusion proteins containing core protein amino acids 124 to 152, 124 to 173, 152 to 173, or 72 to 111. These fusion proteins demonstrated distribution profiles similar to that of GFP alone. We also tested GFP-core-E1 fusions, which are processed at the C terminus of the core by signal peptidase and signal peptide peptidase (19, 30). GFP-core fusions expressed from pGFP/72–382 and pGFP/112-382 were detected in the ER and mitochondrial fractions. The fusion expressed from pGFP/152–382 was not identified in these fractions.

We further analyzed subcellular localization of the fusion proteins by confocal immunofluorescence microscopy (Fig. 5). As expected, fusions of (72–173)-GFP and (112–152)-GFP exhibited localization to the ER and mitochondria. The patterns of subcellular localization of these fusions are indistinguishable from that of the full-length core protein, as shown in Fig. 1. Expression of (124–152)-GFP or (112–123)-GFP resulted in widespread diffusion of the fusion in the cell. Thus, these results indicate that the region spanning amino acids 112 to 152 can mediate association of the core protein not only to the ER but also to the mitochondria.

We subsequently examined the submitochondrial localization of the core protein with a protease protection assay. As shown in Fig. 6A, HCV core protein localized in the mitochondria was completely digested upon treatment with proteinase K for 30 min at 0°C. Under identical conditions, a marker specific for the mitochondrial outer membrane, Tom20, was also observed to disappear, whereas digestion of a mitochondrial inner membrane marker, Tim17, was not observed. These findings confirm that HCV core protein is localized to the mitochondrial outer membrane.

The predicted secondary structure of the region, amino acids 72 to 173, is shown in Fig. 6B. The presence of a long helical segment, lying between amino acids 116 and 134, and two short α-helices (amino acids 146 to 152 and amino acids 155 to 159) were predicted. The results of the cell fractionation assay and confocal microscopy with a series of deletion mutants shown in Fig. 4 and 5 suggest that an α-helix between amino acids 116 and 134 may be required for associating the core protein with the ER and the mitochondrial outer membrane. When amino acids 117 to 134 are portrayed as a helical wheel, we found an amphipathic structure with hydrophobic residues on one side and polar residues on the other side of the α-helix (Fig. 6C), which is often observed in membrane-associated proteins. This helical conformation might be important for directing the core protein to the ER and mitochondrial outer membranes.

Nuclear localization of the HCV core protein is mediated by a bipartite NLS, possibly via an importin-dependent pathway. Although HCV core protein is mainly localized within the cytoplasm, it is also found in the nucleus, as shown in Fig. 3.

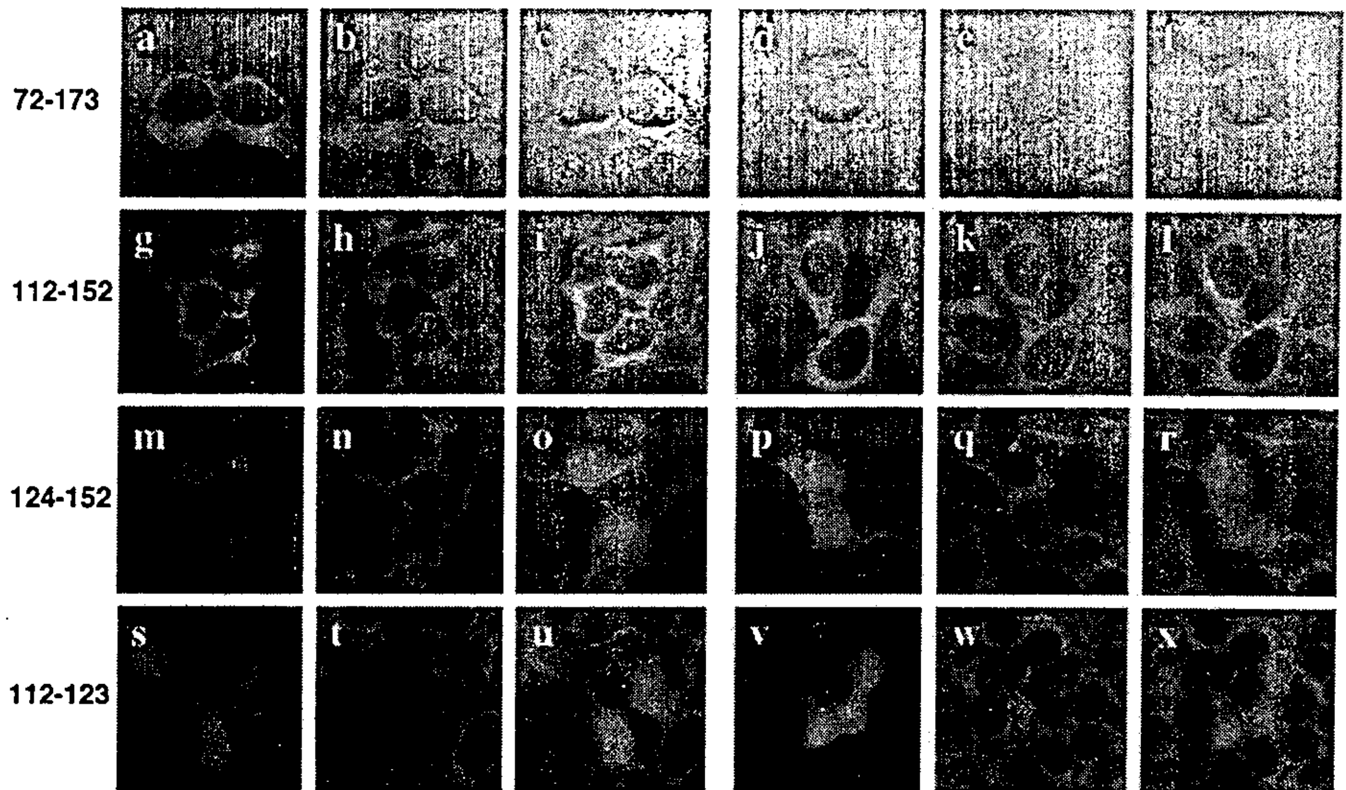

FIG. 5. Confocal analysis of double staining for core-GFP fusion protein and ER or mitochondria. 293T cells transfected with core-GFP expression plasmids (72–173, 112–152, 124–152, and 112–123) were allowed to express the plasmid for 2 days. Transfected cells were fixed directly (a to c, g to i, m to o, and s to u) or fixed after loading with Mitotracker (d to f, j to l, p to r, and v to x). After permeabilization with Triton X-100, a goat anticalregulin antibody was used for ER staining. The red signals corresponding to the ER were obtained with a rhodamine-conjugated rabbit anti-goat IgG secondary antibody (b, h, n, and t). Mitochondria were stained with the mitochondrion-selective dye Mitotracker (e, k, q, and w). Overlay resulted in yellow signals indicative of colocalization (c, f, i, l, o, r, u, and x).

The results of previous studies demonstrate that the N-terminal region of the core protein is responsible for nuclear targeting. It contains three clusters of basic amino acid residues that represent putative consensus motifs for NLS sequences PKPQRKTKR (amino acids 5 to 13), PRRGPR (amino acids 38 to 43), and PRGRRQPIPKARRP (amino acids 58 to 71) (6, 48). Nuclear targeting is generally governed by a family of transporters or cytosolic receptor proteins, known as importins or karyopherins, which function in concert with a guanine nucleotide-binding protein named Ran and other regulatory proteins such as NTF2/p10. Conventional NLS-dependent nuclear targeting occurs when importin-α recognizes the NLS sequence, mediating binding to importin-β1, after which the trimeric complex translocates to the nucleus (12).

In order to determine whether the putative NLS motifs identified within the core protein sequence are capable of binding to importin-α, we examined the in vitro interaction between bacterially expressed GST-fused core protein and ^{35}S-labeled importin-α with a GST pulldown assay. We then substituted lysine and arginine residues of one or more of the putative NLS motifs of the core protein (all contained within the first 71 amino acids of the N terminus) with alanine and fused the resultant constructs with GST, as shown schematically in Fig. 7A. As shown in Fig. 7B (upper panel), importin-α was pulled down by a GST fusion protein containing wild-type core (amino acids 1 to 71) protein but not with GST alone, suggesting that direct binding occurs between the core protein and importin-α. Importin-α was also pulled down by GST-core fusion proteins containing substitutions in one or two NLS motifs (NLS/m1, NLS/m2, NLS/m3, NLS/m4, NLS/m5, and NLS/m6). However, importin-α was not pulled down by GST-core fusion proteins containing alanine substitutions in all three NLS motifs (NLS/m7). It should be noted that similar amounts of GST fusion proteins were used for each of the in vitro pulldown assays, followed by SDS-PAGE and Coomassie brilliant blue staining (Fig. 7B, lower panel). These results demonstrated that all three putative NLS motifs of the N-terminal region of the core protein can mediate binding to importin-α, which suggests that nuclear translocation of the core protein occurs via an importin-dependent pathway (12).

The interaction between the core and importin-α was further analyzed by a colocalization assay (Fig. 7C). The GFP fusion containing the wild-type core (amino acids 1 to 71) was well colocalized with HA-importin-α; distribution of the two proteins showed similar nuclear staining patterns, confirming the presence of a functional NLS sequence(s) within the core protein. In contrast, NLS/m4, with substitutions in two NLS motifs, was partly colocalized with HA-importin-α near or around the nuclear membrane, suggesting that NLS motif double mutants bind to importin-α but their binding efficiency is lower than that of wild-type core protein.

Finally, we examined the subcellular localization of core

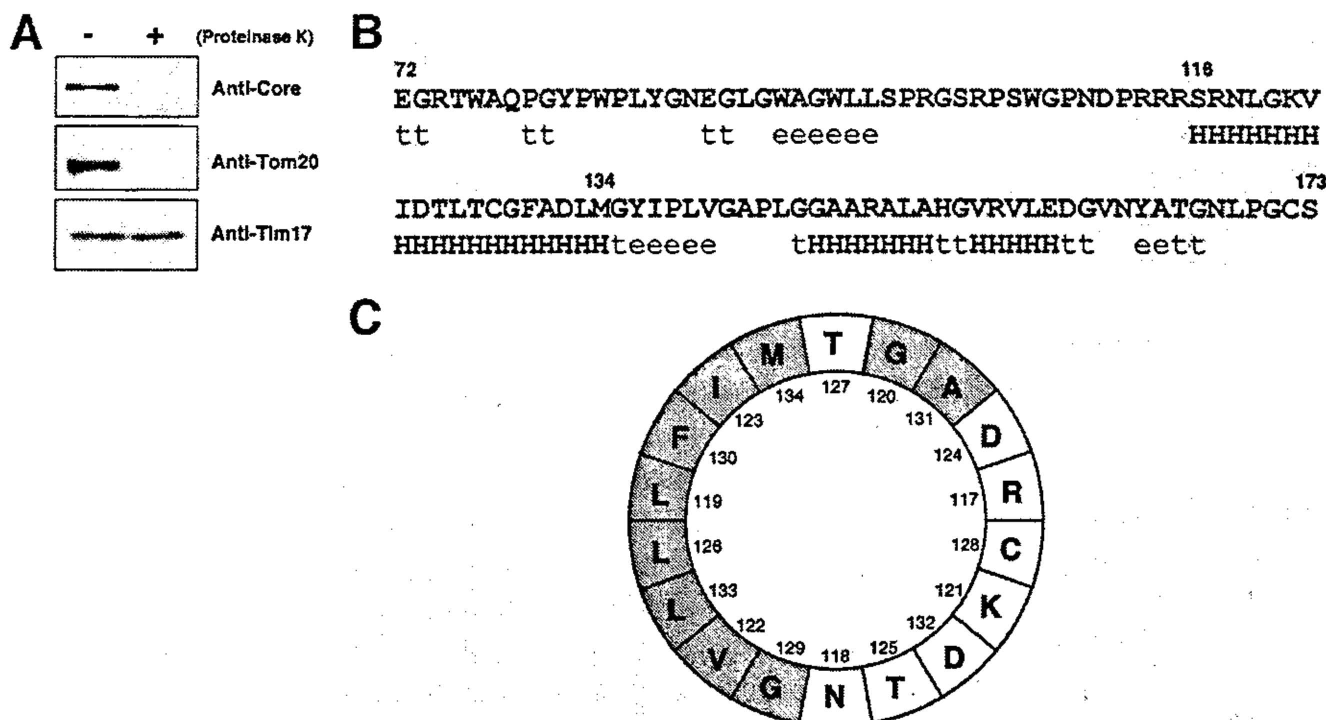

FIG. 6. (A) Protease protection assay. A mitochondrial fraction isolated from cells expressing the core protein was treated with proteinase K (+) as described in Materials and methods. The sample as well as the nontreated fraction (−) were subjected to immunoblotting with a monoclonal antibody against either HCV core, Tom20, or Tim17. (B) Protein sequence and predicted secondary structure of HCV core, amino acids 72 to 173. The secondary structure prediction was obtained with the self-optimized prediction method, a computer program on the internet (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = /NPSA/npsa_sopm.html). H, α-helix; t, turn; e, extension. (C) α-Helical plot of amino acids 117 to 134 of the core protein. In the helical wheel plots, the gray shading represents apolar and hydrophobic residues; and the white represents polar residues.

protein expressed by the wild-type and NLS mutants (Fig. 7D). As expected, a fusion protein containing wild-type core protein (amino acids 1 to 71) and GFP was localized exclusively to the nucleus. Core proteins from three fusion proteins containing substitutions in each NLS motif (NLS/m1, NLS/m2, and NLS/m3) were detected primarily in the nucleus. Weak fluorescence was also observed in the cytoplasm, suggesting that these mutations caused a slight reduction in the efficiency of nuclear translocation. On the other hand, two or three NLS motif substitution mutations (NLS/m4, NLS/m5, NLS/m6, and NLS/m7) completely abolished nuclear translocation, resulting in a diffuse distribution of core protein, similar to that of GFP alone. Although it is likely that all three putative NLS motifs play a role, the above results suggest that at least two of the three putative NLS motifs are prerequisite for efficient nuclear translocation of the core protein.

DISCUSSION

HCV core protein is released from the viral polyprotein by a host protease(s) within the ER membrane at a signal peptide sequence lying between the core and envelope (E1) proteins (16, 41). Subsequently, the signal peptide is further processed by an intramembranous protease called signal peptide peptidase (38, 53). This mature form of the core protein is then released and undergoes subcellular trafficking (30, 53). The core protein localizes mainly to the ER, mitochondria, and lipid droplets. Some reports also describe localization of the core protein to the nuclei of hepatocytes in HCV-infected patients (10), transgenic mice (34), and cultured cells expressing viral polyproteins (56). Although it has been reported which sequence motifs are responsible for localization of the HCV core protein to lipid droplets and nuclei, it is uncertain which sequences target the core protein to the ER and to mitochondria. In this study, we identified sequences related to localization of the mature core protein to the ER and to mitochondria.

Through heterologous expression of core-GFP fusion proteins containing a series of deletions, we determined that a sequence extending from amino acids 112 to 152 of the core protein is required for its localization at the mitochondrial outer membrane. Translocation of nucleus-encoded mitochondrial proteins is usually dependent on N-terminal sequences, referred to as mitochondrial targeting sequences (37). However, it is also true that a significant proportion of mitochondrial proteins lack these N-terminal mitochondrial targeting sequences. Specifically, a number of outer membrane proteins do not have cleavable sequences at their N termini; rather, they are targeted to mitochondria by means of internal or C-terminal signals (31).

Since it has been reported that amino acid sequences required for targeting to the outer mitochondrial membrane form a highly hydrophobic α-helical wheel, as seen in A-kinase associated protein 84/12 (4) and NADH-cytochrome *b* reduc-

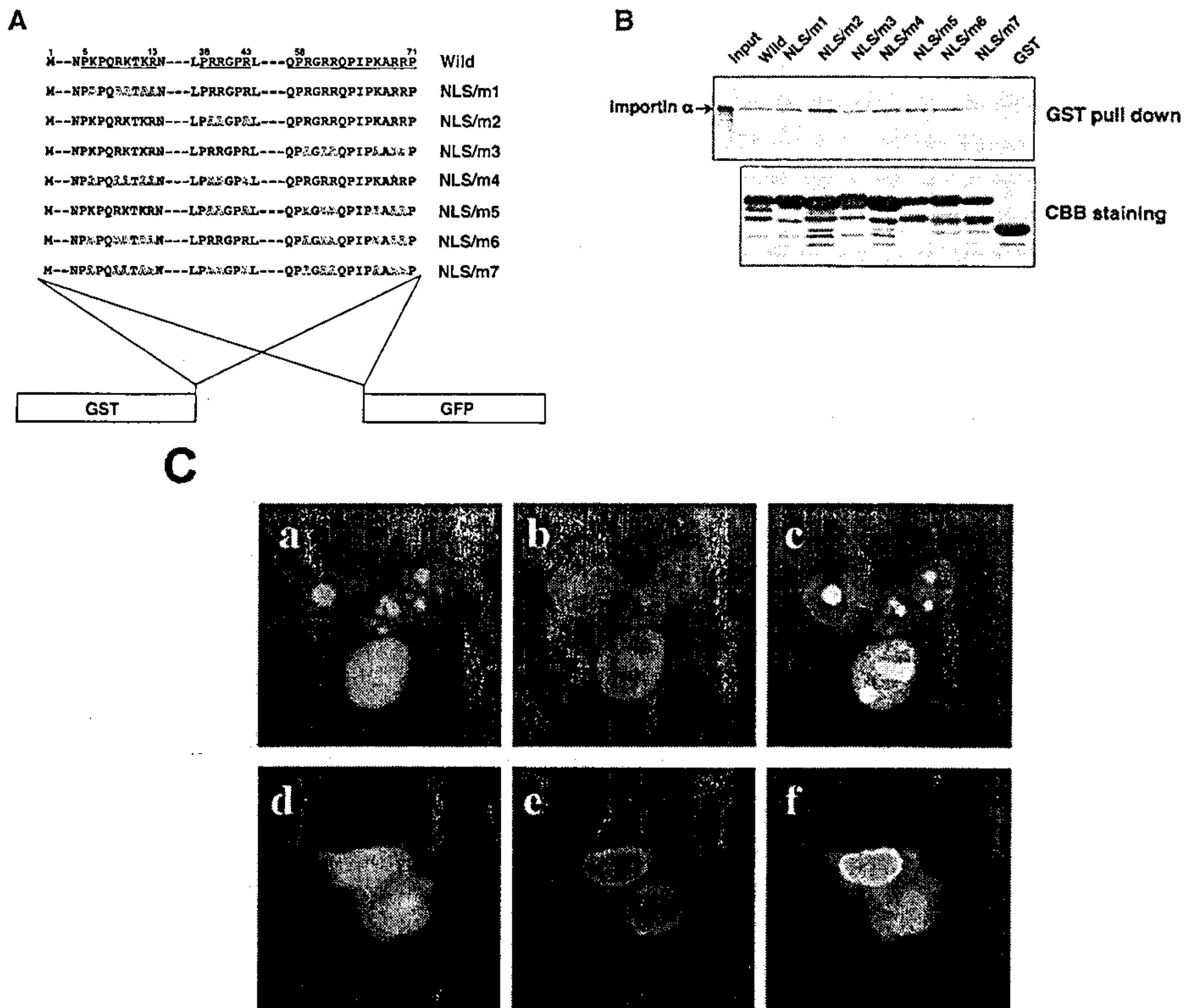

FIG. 7. Mutational analyses of NLS motifs in HCV core protein. (A) Schematic structures of fusion proteins and amino acid sequences corresponding to putative NLS motifs, three basic clusters (underlined) in the core protein. Two series of constructs fused with either GFP or GST were created. The mutated basic residues are indicated with outline letters. (B) GST pulldown assay. Equal amounts of GST fusions as described in A or GST alone was immobilized on glutathione-Sepharose 4B beads and incubated with in vitro-translated, [^{35}S]methionine-labeled importin-α. Bound material was separated by SDS-PAGE, and the amount of importin-α bound was detected by autoradiography. Direct electrophoretic separation of in vitro translation products served as a control (input). Coomassie brilliant blue staining of GST fusions and GST alone are shown in the bottom panel. (C) Confocal analysis of double staining for core-GFP fusion protein and HA-importin-α. 293T cells transfected with the wild-type core (1–71)-GFP (a to c) or NLS/m4 (d to f) expression plasmid and pCAG-HA-imp were allowed to express for 2 days. After the cells were fixed and permeabilized, they were incubated with a mouse anti-HA antibody. The red signals corresponding to HA-importin-α were obtained with a rhodamine-conjugated goat anti-mouse IgG secondary antibody (b and e). Overlay resulted in yellow signals indicative of colocalization (c and f). (D) Subcellular localization of GFP fusion proteins. GFP fusions with and without substitution mutations in the NLS motifs of the core protein as described in A were expressed in 293T cells. GFP images of the fixed cells were recorded.

tase (14), a predicted structure of an amphipathic α-helix present between amino acids 116 and 134 (Fig. 6B and C) possibly plays a role in directing the core protein to the mitochondrial outer membrane. Sequence comparisons demonstrate conservation of the amino acid sequence and secondary structure of the region, amino acids 112 to 152, among a variety of HCV isolates, including the infectious H77c clone (55), as well as a full-length adaptive replicon (3). To gain insight into the significance of the secondary structure of the region in targeting to the mitochondria, further structural and biochemical analyses are needed.

The association of HCV core protein with the mitochondrial membrane suggests that the core protein has the ability to modulate mitochondrial function, possibly by altering the permeability of the mitochondrial membrane. The core protein induces the production of cellular reactive oxygen species in

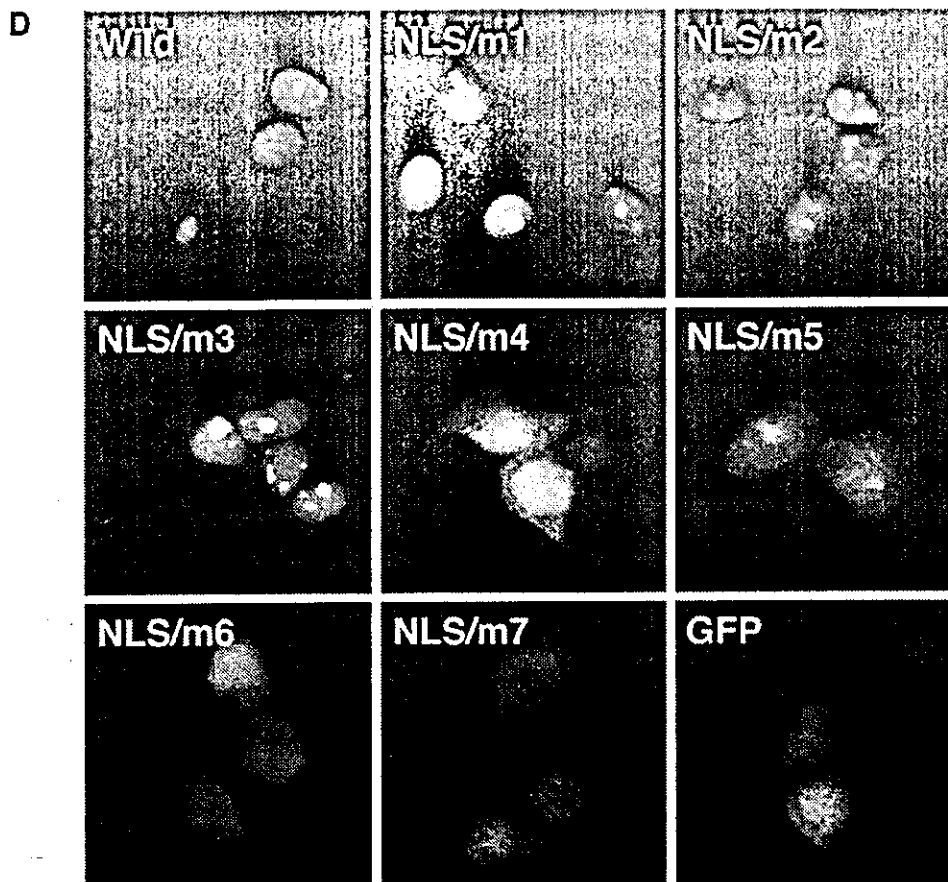

FIG. 7—*Continued.*

the livers of core-transgenic mice and in core-expressing cell lines (35). Reactive oxygen species, predominantly generated in mitochondria, induce genetic mutations and act as secondary messengers to regulate a variety of cellular functions, including gene expression and proliferation (1). Although the molecular mechanism by which core protein induces reactive oxygen species production is still unclear, HCV core protein is known to impair the mitochondrial electron transfer system (35). The core protein may also modulate apoptosis, since mitochondria play a major role in regulating programmed cell death. Expression of HCV proteins, including the core protein, suppresses the release of cytochrome *c* from mitochondria to the cytoplasm in HCV-transgenic mice, thus inhibiting Fas-mediated apoptosis (27).

Okamoto et al. recently reported that not only the C-terminal signal sequence but also amino acids 128 to 151 are required for ER retention of the core protein by using a series of N-terminally truncated core protein constructs (38). Here, in this study, we further showed that amino acids 112 to 152 mediate association of the core protein with the ER in the absence of the C-terminal signal sequence. Hope and McLauchlan demonstrated that the central domain of the core protein, amino acids 119 to 174, is important for association with lipid droplets (17). They also showed that this corresponding domain is shared with GB virus B, which is most closely related to HCV, but not with either pestiviruses or flaviviruses (18). It appears that the 41 residues identified as the sequence mediating association with the ER membrane in the present study are crucial for directing the core protein to lipid droplets, since the surface of lipid droplets must derive from the cytoplasmic side of the ER membrane.

The HCV core protein contains NLS sequences which are composed of three stretches of sequences rich in basic residues. These sequences were originally identified by experiments with fused forms of wild-type and mutated core proteins with β-galactosidase (6, 48). C-terminally truncated versions of the core protein localize exclusively to the nucleus (48). A fraction of the core protein is detected in the nucleus even when full-length HCV core gene is expressed (Fig. 2) and as described (34, 56). However, it is difficult to demonstrate clearly the nuclear localization of the core protein by immunofluorescence, presumably because of the instability of nuclearly localized core protein (49, 33). We only observed a nuclear staining pattern of the matured core protein after adding proteasome inhibitors to the culture (33).

Generally, NLS sequences fall into two categories; (i) monopartite NLSs, which contain a single cluster of basic residues, and (ii) bipartite NLSs, which contain two clusters of basic residues separated by an unconserved linker sequence of variable length (reviewed in reference 12). Nuclear translocation of an NLS-containing cargo protein is initiated when the soluble import receptor (importin) recognizes the NLS-contain-

ing protein within the cytoplasm. Importin-α contains an NLS-binding site(s), and importin-β docks importin-cargo complexes to the cytoplasmic filaments of a nuclear pore complex, after which translocation occurs through the nuclear pore. Thus, importin-α functions as an adaptor between the bona fide import receptor and the NLS-containing protein.

We further characterized the NLS of the core protein and found that each of the NLS motifs of the core protein is able to bind to importin-α and that at least two NLS motifs are required for efficient nuclear distribution of the core protein in cells. It appears that double mutations among three NLS motifs decrease the ability of the core protein to bind importin-α. These observations suggest that the binding of the double mutants with importin-α leads to no or little active translocation of the core protein into the nucleus. The double mutations may also block subsequent interactions with importin-β1, GTPase Ran, and/or NTF2/p10, which are required for translocation through the nuclear pore complexes.

The findings obtained in this study suggest that HCV core protein NLS motifs have a bipartite function. Crystallographic studies of monopartite (e.g., simian virus 40 large T antigen) and bipartite (e.g., nucleoplasmin) NLSs show that the basic residue clusters of bipartite NLSs occupy separate binding sites on importin-α. In contrast, while monopartite NLSs can bind to the same sites as bipartate NLSs on importin-α, they mainly bind to the N-terminal binding site, which is referred to as the major binding site on importin-α (9, 11). A recent report describes an importin-α variant with a mutation in the major site which results in decreased ability to bind both monopartite and bipartite NLSs. Another variant with a mutation in the minor site exhibits decreased binding only to bipartite NLS-containing proteins, making importin-α nonfunctional in vivo (22). Thus, we favor a model in which the core protein bipartite NLS, composed of any two of the three basic clusters, occupies both major and minor binding sites on importin-α, resulting in efficient nuclear translocation. Importin-α may be equally accessible to all clusters, given their close proximity to one another, as well as the distinct conformational flexibility of the ≈70-residue N-terminal region of the core protein.

With regard to the molecular mechanisms participating in nuclear localization of the core protein, Moriishi et al. found that PA28γ is involved in nuclear localization of the core protein. Interaction of the core protein with PA28γ plays an important role in retention of the core protein in the nucleus (33). Furthermore, in yeast cells, nuclear transport of the core protein requires the activity of the small GTPase Ran/Gsp1p and is mediated by Kap123p, but neither importin-α nor importin-β is involved (20). Differences in nucleocytoplasmic transport between yeast and mammalian cells might explain the inconsistencies observed in the present study. Further experiments are required to characterize the exact nature of the interaction between the core protein and components of the nuclear import machinery, particularly in cells where HCV is replicating.

In conclusion, the mature HCV core protein has an internal 41-amino-acid sequence mediating association of the viral protein with the ER and mitochondria. We also provide evidence for a novel class of bipartite NLS contained within the core protein, which comprises two of three basic motifs, thus enabling efficient nuclear targeting. Multiple functional domains influence the subcellular localization of the core protein, which ultimately depends on the balance of the respective signals.

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Intramembrane Proteolysis and Endoplasmic Reticulum Retention of Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein is suggested to localize to the endoplasmic reticulum (ER) through a C-terminal hydrophobic region that acts as a membrane anchor for core protein and as a signal sequence for E1 protein. The signal sequence of core protein is further processed by signal peptide peptidase (SPP). We examined the regions of core protein responsible for ER retention and processing by SPP. Analysis of the intracellular localization of deletion mutants of HCV core protein revealed that not only the C-terminal signal-anchor sequence but also an upstream hydrophobic region from amino acid 128 to 151 is required for ER retention of core protein. Precise mutation analyses indicated that replacement of Leu^{139}, Val^{140}, and Leu^{144} of core protein by Ala inhibited processing by SPP, but cleavage at the core-E1 junction by signal peptidase was maintained. Additionally, the processed E1 protein was translocated into the ER and glycosylated with high-mannose oligosaccharides. Core protein derived from the mutants was translocated into the nucleus in spite of the presence of the unprocessed C-terminal signal-anchor sequence. Although the direct association of core protein with a wild-type SPP was not observed, expression of a loss-of-function SPP mutant inhibited cleavage of the signal sequence by SPP and coimmunoprecipitation with unprocessed core protein. These results indicate that Leu^{139}, Val^{140}, and Leu^{144} in core protein play crucial roles in the ER retention and SPP cleavage of HCV core protein.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (5, 19) and has been estimated to infect more than 170 million people throughout the world (15). Symptoms of persistent HCV infection extend from chronic hepatitis to cirrhosis and finally to hepatocellular carcinoma (18, 42). HCV belongs to the genus *Hepacivirus* in the family *Flaviviridae* and possesses a viral genome consisting of a single, positive-strand RNA with a nucleotide length of about 9.4 kb (6, 48). The genome encodes a large precursor polyprotein of approximately 3,000 amino acids (6, 17). The polyprotein is processed co- and posttranslationally into at least 10 viral proteins by host and viral proteases (2, 6, 10, 45). The structural proteins of HCV are located in the N-terminal one-fourth of the polyprotein and are cleaved by host membrane proteases (10, 44). Comparison with other flaviviruses suggests that HCV core protein forms the nucleocapsid, which is surrounded by the envelope containing glycoproteins E1 and E2 (6, 48). Functional analyses suggest that HCV core protein has regulatory roles in host cellular functions. In tissue culture systems, HCV core protein regulates signaling pathways and modulates apoptosis (4, 29, 40, 41, 46, 54, 55). Moreover, transgenic mice expressing HCV core protein developed liver steatosis and thereafter hepatocellular carcinoma (34, 36). Thus, it has been suggested that HCV core protein is a multifunctional molecule that acts as a structural protein but is also involved in the pathogenesis of hepatitis C. HCV core protein has two major forms, p23 and p21 (16, 25, 31, 43, 53). HCV core protein p23 represents a 191-amino-acid product in which the C-terminal hydrophobic region also acts as a signal sequence for E1. HCV polyprotein is cleaved between residues 191 and 192 by host signal peptidase to generate C-terminal and N-terminal polypeptides encompassing the core and E1 proteins, respectively. For the full maturation of HCV core protein, the C-terminal signal-anchor sequence was thought to be further processed by an unidentified microsomal protease (25, 30, 31, 43, 53), and the 21-kDa isoform of core protein is predominantly detected both in cultured cells by transfection with expression plasmid and in viral particles obtained from sera of patients with hepatitis C (53). These results suggest that p21 is the mature form of HCV core protein (53). Immunostaining revealed that most HCV core protein is distributed diffusely throughout the cell, probably in the endoplasmic reticulum (ER) (31, 53). However, a minor population was observed in the nucleus (53).

Recently, a presenilin-related aspartic protease, signal peptide peptidase (SPP), was identified (50). SPP is located in the ER membrane and promotes intramembrane proteolysis of signal peptides. The chemical compound $(Z\text{-}LL)_2$-keton inhibits processing of signal peptides by SPP, and it was shown to suppress intramembrane proteolysis of major histocompatibility complex class I molecules, preprolactin, HCV core protein, and others (21, 30, 51). Replacement of Asp^{265} with Ala in SPP resulted in a loss of catalytic function, although this mutant could bind to TBL_4K, a derivative of $(Z\text{-}LL)_2$-keton (50). HLA-A was processed into yeast microsomes following the addition of wild-type SPP but not mutant SPP, suggesting that SPP interacts with HLA-A (50). Processing of the signal sequence of HCV core protein by SPP was inhibited by the

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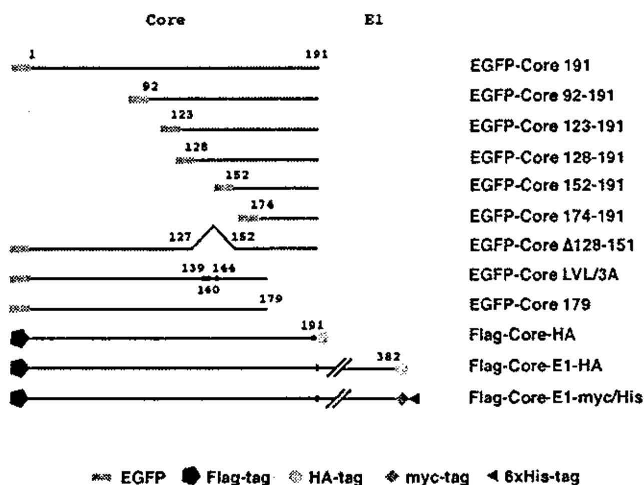

FIG. 1. Expression plasmids used in this study. The genes encoding HCV proteins and their mutants were cloned into pcDNA3.1FlagHA, pcDNA3.1/*myc*-His C, or pEGFP-C3 as described in Materials and Methods. Other plasmids are described in the text or in the other figure legends.

addition of $(Z\text{-}LL)_2$-keton, and Ser^{183} and Cys^{184} in the signal sequence of core protein were demonstrated to be important for flexibility and intramembrane proteolysis by SPP (23). Signal sequences generally have a tripartite structure, including a central hydrophobic H region and hydrophilic N- and C-terminal flanking regions (28). SPP recognizes the N- and C-terminal regions and cleaves in the middle of the H region (28). Mutational analyses suggested that the flexibility of signal peptides is generally required for substrate recognition of SPP (23). SPP contains the aspartic protease motifs YD and LGLGD, which are located in the predicted transmembrane region, and it is thought to cleave type II (N terminus in the cytosol and C terminus in the lumen)-oriented substrates (50). However, the effect of the cytoplasmic region of type II membrane substrates on intramembrane proteolysis by SPP is not known. In this study, we examined the regions of HCV core protein that are essential for ER retention and intramembrane cleavage by SPP.

MATERIALS AND METHODS

Plasmids. For expression of enhanced green fluorescence protein (EGFP)-fused HCV core proteins in culture cells, the core protein-coding region was amplified by PCR from cDNA encoding full-length HCV polyprotein type 1b (1). The PCR products were subcloned into SalI and BamHI sites 3′ of the EGFP-coding region of pEGFP-C3 (Clontech, Palo Alto, Calif.). The cDNA fragments encoding amino acids 1 to 191, 1 to 179, 92 to 191, 123 to 191, 128 to 191, 152 to 191, and 174 to 191 of HCV core proteins were amplified by PCR and then introduced into pEGFP-C3; these constructs are designated EGFP-Core 191, EGFP-Core 179, EGFP-Core 123-191, EGFP-Core 128-191, EGFP-Core 152-191, and EGFP-Core 174-191, respectively. The genes encoding core proteins with the region between amino acids 128 and 151 deleted and replacement of Leu^{139}, Val^{140}, and Leu^{144} with Ala were generated by the method of splicing by overlap extension (11, 14, 49) and introduced into pEGFP-C3; these constructs are designated EGFP-Core Δ128-151 and EGFP-Core LVL/3A, respectively (Fig. 1).

Fragments encoding Flag and hemagglutinin (HA) tags were inserted at both ends of the multicloning site of pcDNA3.1 (pcDNA3.1FlagHA). PCR products encoding either HCV core protein alone, core protein followed by E1 (Core-E1), or their mutants were cloned into pcDNA3.1FlagHA, resulting in plasmids encoding recombinant proteins sharing Flag and HA tags at the N and C termini, respectively (Fig. 1). In Flag-Core-HA and its derived mutants, Ala^{191} was replaced by Arg to avoid processing by signal peptidase for determination of cleavage by SPP, as previously shown for the processing of the E1-E2 junction (7). In addition, the region encoding Flag-Core-E1 or its mutants was cleaved from pcDNA3.1FlagHA constructs and then introduced between the SacI and XhoI sites of pcDNA3.1/*myc*-His C (Invitrogen Corp., Carlsbad, Calif.). The resulting plasmids encode HCV proteins sharing Flag and myc/His epitopes at the N and C termini, respectively (Fig. 1). Genes encoding core protein with a single amino acid (Leu^{139}, Val^{140}, or Leu^{144}), double amino acids (Leu^{139} and Val^{140}, Leu^{139} and Leu^{144}, or Val^{140} and Leu^{144}), or triple amino acids (Leu^{139}, Val^{140}, and Leu^{144}) replaced with Ala were generated by splicing by overlap extension and introduced into pcDNA3.1FlagHA and pcDNA3.1/*myc*-His C (Fig. 1; see Fig. 4).

The genes encoding the ER-targeting and ER retrieval sequences of calreticulin fused with DsRed at the N and C termini, respectively (8, 37, 39), were inserted between the EcoRV and XbaI sites of pcDNA3.1 (pcDNA ER-DsRed) to visualize the ER in culture cells. This recombinant protein is designated ER-DsRed in this study.

Cloning of SPP. The cDNA encoding SPP was amplified from human liver mRNA (Clontech) by reverse transcription-PCR and cloned into T-vector prepared from pBluescript II SK(−) (27). The gene encoding SPP with an attached HA tag and ER retrieval signal, KEKK, at the C terminus (SPP-HAER) was cloned into pcDNA3.1 to eliminate the possibility that the HA tag suppresses the endogenous ER retrieval signal of SPP. SPP-HAER was colocalized with ER-DsRed on the ER membrane and glycosylated upon transfection into cells (data not shown).

Subcellular localization of wild-type and mutant HCV core proteins. HeLa cells were maintained in the Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HeLa cells were seeded on an eight-well chamber slide at 2×10^4 cells per well 24 h before transfection. The cells were transfected with the various plasmids by lipofection with Lipofectamine 2000 (Invitrogen). To determine protein subcellular localizations, transfected cells were fixed with phosphate-buffered saline (PBS) containing 3% paraformaldehyde at 18 h posttransfection and then observed with a confocal laser-scanning microscope (Bio-Rad, Tokyo, Japan). To confirm subcellular localization of the core proteins, transfected cells were fractionated with a subcellular proteome extraction kit (Calbiochem, Darmstadt, Germany). Stepwise extraction resulted in four distinct fractions, which contain mainly cytosolic, membrane-organelle, nuclear, and cytoskelton proteins, respectively. Each fraction was precipitated with trichloroacetic acid and analyzed by immunoblotting, and the densities of the bands were measured with Multi Gauge version 2.2 (Fujifilm, Tokyo, Japan).

Immunoblotting. After transfection, 293T cells were harvested, washed twice with PBS, and lysed in 20 mM Tris-HCl (pH 7.4) containing 135 mM NaCl, 1% Triton X-100, and 10% glycerol (lysis buffer) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and 1 mM Na_2VO_3. The lysate was centrifuged at 6,500 × *g* for 5 min at 4°C. The resulting supernatants were subjected to sodium dodecyl sulfate (SDS)–13.5% polyacrylamide gel electrophoresis. The separated proteins were electroblotted onto a Hybond-P polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, N.J.). These membranes were blocked with PBS containing 5% skim milk and 0.05% Tween 20 (Sigma, St. Louis, Mo.) and incubated with mouse monoclonal anti-Flag M2 (Sigma), anti-HA 16B12 (HA.11; BabCO, Richmond, Calif.), or monoclonal mouse anti-His_6-AD1.1.10 (Genzyme/Techne, Tokyo, Japan) immunoglobulin G (IgG) at room temperature for 30 min and then with horseradish peroxidase-conjugated anti-mouse IgG antibody at room temperature for 30 min. Immunoreactive bands were visualized by using the enhanced chemiluminescence Super Signal West Femto substrate (Pierce, Rockford, Ill.).

Immunoprecipitation. Immunoprecipitation analysis was carried out as described previously (32). Plasmids were transfected into 293T cells by lipofection. Transfected cells were harvested at 18 h posttransfection and lysed in lysis buffer with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid (CHAPSO) (Dojindo, Kumamoto, Japan). Cell lysates were incubated with monoclonal anti-HA, anti-Glu-Glu (anti-EE) (BabCO), or anti-Flag antibody at 4°C for 1.5 h and then with protein G–Sepharose CL-4B (Amersham Bioscience) at 4°C for 1.5 h. After centrifugation at 6,500 × *g* for 3 min at 4°C, the pellets were washed five times with lysis buffer. Immunoprecipitates were subjected to immunoblotting.

Deglycosylation. Plasmids encoding core and E1 proteins were transfected into 293T cells by lipofection, and cell lysates were immunoprecipitated with anti-HA antibody at 18 h posttransfection. Immunoprecipitates were eluted from protein G–Sepharose CL-4B in 0.5% SDS and 1% 2-mercaptoethanol and digested with endo-β-*N*-acetylglucosaminidase H (Endo H) or peptide-*N*-glycosidase F

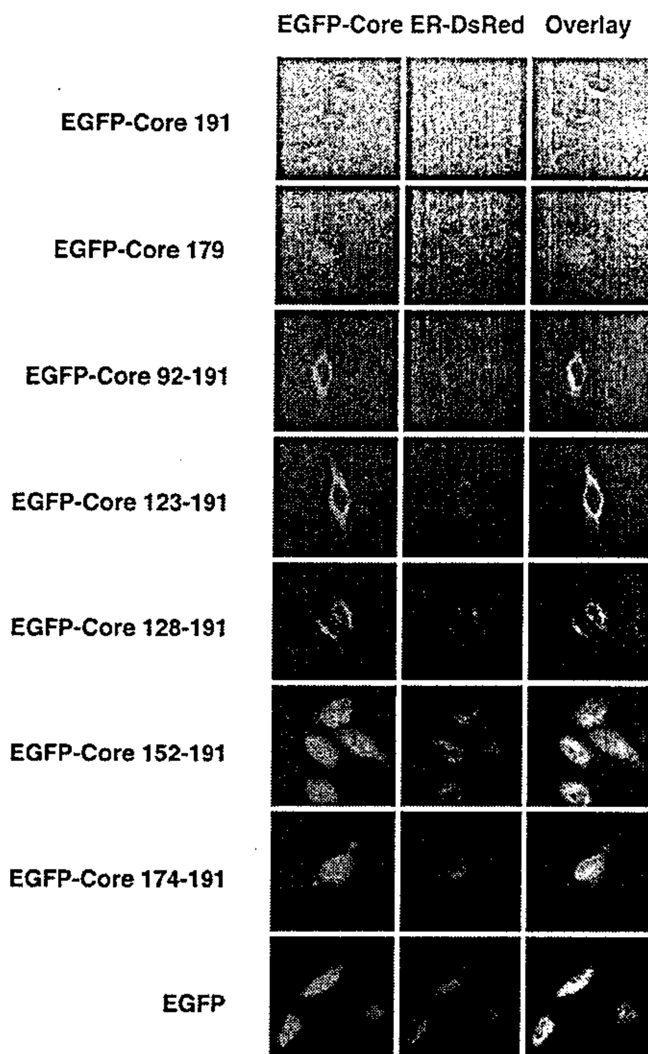

FIG. 2. Intracellular localization of EGFP-Core mutants. EGFP-Core and its deletion mutants were coexpressed with ER-DsRed in HeLa cells, and the localization of core proteins was examined by confocal microscopy.

(PNGase F) according to the protocol of the manufacturer (Roche, Mannheim, Germany). The resulting mixtures were subjected to immunoblotting.

RESULTS

Region required for ER retention of HCV core protein. To determine the regions within HCV core protein that are responsible for ER retention, EGFP-fused, N-terminally truncated HCV core protein (Fig. 1) was coexpressed with the ER marker ER-DsRed. EGFP-Core 191 colocalized with ER-DsRed to the ER (Fig. 2), whereas EGFP-Core 179 was localized primarily to the nucleus as reported previously (3, 33, 43, 47), suggesting that the C-terminal signal sequence is essential for anchoring HCV core protein to the ER membrane. However, EGFP-Core 174-191 exhibited diffuse staining similar to that of EGFP, suggesting that the signal sequence alone is not sufficient for ER localization. EGFP-Core 92-191, EGFP-Core 123-191, and EGFP-Core 128-191 were colocalized with ER-DsRed in the ER, but EGFP-Core 152-191 stained similarly to EGFP-Core 174-191 and EGFP. These data suggest that not only the C-terminal signal sequence but also the region from amino acids 128 to 151 is required for ER retention of HCV core protein.

Region essential for processing of the signal sequence of HCV core protein by SPP and signal peptidase. Based on hydrophobicity and a cluster of basic amino acids, HCV core protein was proposed to possess three regions (domains 1 to 3) (Fig. 3A, upper panel) by Hope and McLauchlan (12). To assess the involvement of the region encompassing amino acids 128 to 151 in proteolysis of the signal sequence of HCV core protein by signal peptidase and SPP, three hydrophobic amino acids, Leu^{139}, Val^{140}, and Leu^{144}, in the most hydrophobic peak in domain 2 were replaced with Ala to reduce hydrophobicity, and Ala^{191} was replaced with Arg to eliminate processing by signal peptidase (Fig. 3A, lower panel). When a wild-type Flag-Core-HA construct was expressed in 293T cells, a single band of 23 kDa was detected by blotting with anti-Flag, but not with anti-HA, suggesting that the HA-fused signal sequence was properly processed by SPP and that Flag-core protein of 23 kDa was generated (Fig. 3B, lanes 2 and 11). In cells expressing the substitution mutants, 25- and 23-kDa bands were detected by the anti-Flag antibody (Fig. 3B, upper panel, lanes 3 to 9) and 25-kDa bands were detected by the anti-HA antibody (Fig. 3B, lower panel, lanes 3 to 9), indicating that the 25- and 23-kDa bands correspond to core proteins that are unprocessed and processed by SPP, respectively. Cleavability of the signal sequence of mutant core proteins by SPP was suppressed in accordance with the number of substitutions, and almost no processing of the signal sequence was observed in cells expressing Flag-Core LVL/3A-HA, which has three amino acid substitutions (Fig. 3B, lane 9). These results indicate that Leu^{139}, Val^{140}, and Leu^{144} play crucial roles in the processing of the signal-anchor of HCV core protein by SPP. Furthermore, deletion of the hydrophobic region including amino acids 128 to 151 from HCV core protein completely eliminated processing by SPP, and this species was seen only as a single band of 23.5 kDa which was detected by both the anti-Flag and anti-HA antibodies (Fig. 3B, lane 10). Taken together with the observation that Ala^{180}, Ser^{183}, and Cys^{184} in the signal sequence of HCV core protein of the type 1a Glasgow strain were demonstrated to be essential for SPP proteolysis (13, 23), these results indicate that the hydrophobic region from amino acid 139 to 144 in domain 2 of HCV core protein also participates in the processing of the signal sequence by SPP.

To examine the role of the region from amino acid 139 to 144 in the cleavage of the HCV core protein signal sequence by signal peptidase and SPP in more detail, substitutions of Leu^{139}, Val^{140}, and/or Leu^{144} with Ala were introduced into the Flag-Core-E1-HA polyprotein (Fig. 1). Flag-Core-E1-HA protein was cleaved to the expected molecular mass of 23 kDa of Flag-Core protein by signal peptidase and SPP (Fig. 3C, lanes 2 and 11), whereas slightly larger bands corresponding to a core protein unprocessed by SPP were detected in cells expressing polyproteins possessing mutations within amino acids 139 to 144 (Fig. 3C, lanes 3 to 9). A lack of processing by SPP was detected mainly in core proteins containing double amino acid changes of Leu^{139}, Val^{140}, and/or Leu^{144} to Ala

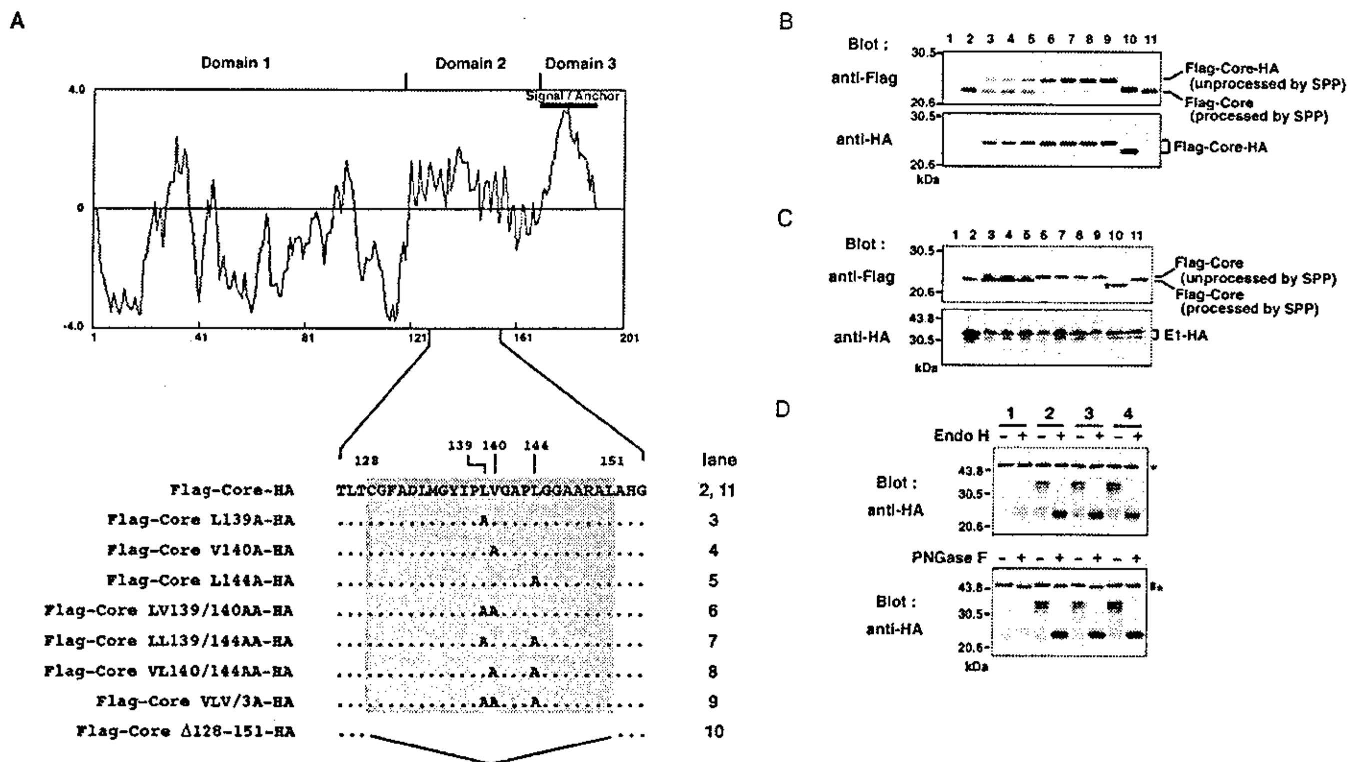

FIG. 3. Identification of the region responsible for processing of the signal sequence of HCV core protein by SPP and signal peptidase. (A) The hydrophobicity profile of HCV core protein was predicted by the method of Kyte and Doolittle (20). Hope and McLauchlan separated the HCV core protein into three regions, domains 1 to 3 (12). Two hydrophobic regions are predicted in the regions from amino acid 128 to 151 and from amino acid 164 to 186 in the C terminus of the HCV core protein. Mutations and deletions in the region from amino acid 128 to 151 of Flag-Core-HA and Flag-Core-E1-HA constructs are indicated. Dots indicate unchanged amino acids. (B) Expression of Flag-Core-HA polyproteins with changes of Ala[191] to Arg in 293T cells. Flag-Core-HA (lanes 2 and 11), Flag-Core L139A-HA (lane 3), Flag-Core V140A-HA (lane 4), Flag-Core L144A-HA (lane 5), Flag-Core LV139/140AA-HA (lane 6), Flag-Core LL139/144AA-HA (lane 7), Flag-Core VL140/144AA-HA (lane 8), Flag-Core LVL/3A-HA (lane 9), and Flag-Core Δ128-151-HA (lane 10) were analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. Cells transfected with an empty plasmid were used as a negative control (lane 1). (C) Expression of Flag-Core-E1-HA mutants in 293T cells. Flag-Core-E1-HA (lanes 2 and 11), Flag-Core L139A-E1-HA (lane 3), Flag-Core V140A-E1-HA (lane 4), Flag-Core L144A-E1-HA (lane 5), Flag-Core LV139/140AA-E1-HA (lane 6), Flag-Core LL139/144AA-E1-HA (lane 7), Flag-Core VL140/144AA-E1-HA (lane 8), Flag-Core LVL/3A-E1-HA (lane 9), and Flag-Core Δ128-151-E1-HA (lane 10) were analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. The asterisk indicates unprocessed Flag-Core Δ128-151. Cells transfected with an empty plasmid were used as a negative control (lane 1). (D) The deglycosylation procedure is described in Materials and Methods. After transfection, cell lysates were immunoprecipitated with anti-HA antibody and immunoprecipitates were digested with Endo H (upper panel) or PNGase F (lower panel). Following digestion, proteins were separated by SDS-polyacrylamide gel electrophoresis, and material from cells transfected with vector (lane 1), Flag-Core-E1-HA (lane 2), Flag-Core LVL/3A-E1-HA (lane 3), and Flag-Core Δ128-151-E1-HA (lane 4) was detected by blotting with anti-HA. Nontreated and Endo H- or PNGase F-treated samples are indicated by − and +, respectively. Asterisks indicate mouse IgG heavy chains.

(Fig. 3C, lanes 6 to 8), and only an unprocessed band was detected in a triple amino acid substitution mutant (Fig. 3C, lane 9) and a deletion mutant lacking amino acids 128 to 151 (Fig. 3C, lane 10). In contrast to the processing of core protein, E1 protein processed from the mutant polyproteins exhibited the same molecular mass of 32 to 35 kDa and the same deglycosylation patterns following digestion with Endo H or PNGase F (Fig. 3D). These results indicate that the internal hydrophobic region from amino acid 139 to 144 of HCV core protein is essential for processing by SPP but not for cleavage of the core-E1 junction by signal peptidase and the subsequent translocation of E1 protein into the ER. It was suggested that signal peptides must be liberated from the precursor protein by cleavage with signal peptidase in order for them to become substrates for SPP (23). Our data indicate that processing by SPP is not a prerequisite for cleavage of the core-E1 junction by signal peptidase.

Amino acid sequence essential for SPP cleavage of the signal sequences of HCV core proteins of genotypes 1a and 1b. Martoglio and colleagues reported that HCV core protein is processed by SPP after cleavage by host signal peptidase and that Ala[180], Ser[183], and Cys[184] residues in the signal sequence of HCV core protein of type 1a Glasgow strain are essential for SPP proteolysis, as they maintain the structure of the breaking α-helix (23, 30). To determine the amino acids essential for SPP cleavage of the signal sequence of type 1b HCV core protein, Flag-Core-E1-HA and its substitution mutants were expressed in 293T cells (Fig. 4). Mutation of one, two, or three amino acids, except for Flag-Core IF176/177AL-E1-HA (Fig. 4B, lane 9), did not affect the processing of the core protein signal sequence. Flag-Core IF176/177AL-E1-HA exhibited the same molecular size as Flag-Core LVL/3A-E1-HA (Fig. 4B, lane 2), suggesting that Ile[176] and Phe[177] in the signal sequence of core protein are essential for cleavage by SPP in our system.

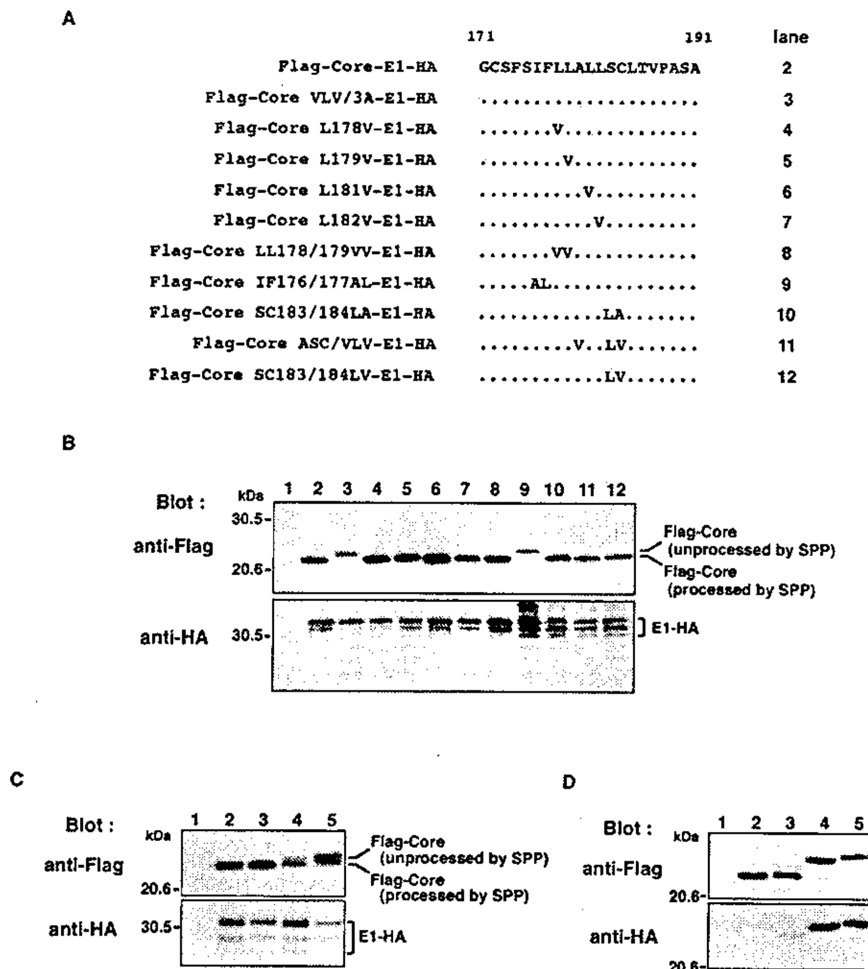

FIG. 4. Amino acid residues essential for SPP cleavage of the HCV core protein signal sequence of genotype 1a and 1b strains. (A) Mutations in the amino acid residues in the signal sequence of Flag-Core-E1-HA are indicated. Dots indicate unchanged amino acids. (B) Flag-Core-E1-HA (lane 2), Flag-Core LVL/3A-E1-HA (lane 3), Flag-Core L178V-E1-HA (lane 4), Flag-Core L179V-E1-HA (lane 5), Flag-Core L181V-E1-HA (lane 6), Flag-Core L182V-E1-HA (lane 7), Flag-Core LL178/179VV-E1-HA (lane 8), Flag-Core IF176/177AL-E1-HA (lane 9), Flag-Core SC183/184LA-E1-HA (lane 10), Flag-Core ASC/VLV-E1-HA (lane 11), or Flag-Core SC183/184LV-E1-HA (lane 12) was expressed in 293T cells. (C) The gene encoding core and E1 polyprotein of the genotype 1a H77c strain of HCV was introduced into pcDNA3.1FlagHA. Flag-H77c Core-E1-HA (lane 2), Flag-H77c Core ASC/VLV-E1-HA (lane 3), Flag-H77c Core LVL/3A-E1-HA (lane 4), or Flag-H77c Core IF176/177AL-E1-HA (lane 5) was expressed in BHK cells. Cell lysates were analyzed by immunoblotting with anti-Flag (upper panel) and anti-HA (lower panel) antibodies. (D) Expression of Flag-Core 191-HA mutants in 293T cells. The gene encoding core protein with a change of Ala^{191} to Arg was introduced into pcDNA3.1FlagHA. Flag-Core-HA (lane 2), Flag-Core ASC/VLV-HA (lane 3), Flag-Core LVL/3A-HA (lane 4), and Flag-Core IF176/177AL-HA (lane 5) were analyzed by immunoblotting with anti-Flag (upper panel) and anti-HA (lower panel) antibodies. Cells transfected with an empty plasmid were used as a negative control (lanes 1 in panels B, C, and D).

However, the triple amino acid substitution (Ala^{180}, Ser^{183}, and Cys^{184}) in the type 1b J1strain (Flag-Core ASC/LVL-E1-HA) (Fig. 4B, lane 11), which is the same as the spmt mutant of the type 1a Glasgow strain (23, 30), did not affect the processing of the signal sequence of HCV core protein by SPP. All derived E1 proteins exhibited a molecular mass of 32 to 35 kDa irrespective of the presence of mutations, and deglycosylation by digestion with endoglycosidases generated uniform 22-kDa bands of E1 proteins (data not shown). These results indicate that Ile^{176} and Phe^{177}, but not Ala^{180}, Ser^{183}, and Cys^{184}, in the signal sequence of type 1b HCV core protein are essential for processing by SPP and confirm that processing of signal sequence by SPP is not required for cleavage by signal peptidase and translocation of E1 protein into the ER. To determine whether the difference in cleavage of signal sequence depends on the genotype of HCV, Ala^{180}, Ser^{183}, and Cys^{184} in the HCV core protein of the genotype 1a H77c strain were replaced with Val, Leu, and Val, respectively. The spmt construct of the type 1a H77c strain did not affect the processing of core and E1 proteins in BHK cells (Fig. 4C, lane 3) and 293T cells (data not shown). In contrast, replacement of Leu^{139}, Val^{140}, and Leu^{144} by Ala and of Ile^{176} and Phe^{177} by Ala and Leu suppressed the processing of the core protein signal sequence of the type 1a H77c strain in BHK cells (Fig. 4C, lanes 4 and 5). These results indicate that three hydrophobic amino acids Leu^{139}, Val^{140}, and Leu^{144} in the hydrophobic peak in domain 2 and the two amino acids Ile^{176} and Phe^{177} in the transmembrane domain play important roles in the in-

tramembrane proteolysis of HCV core protein signal sequence of genotypes 1a and 1b by SPP.

To further examine the cleavage of the signal sequence of HCV core proteins by SPP, we prepared IF176/177AL and the spmt mutant core proteins carrying a substitution of Ala[191] to Arg to avoid processing by signal peptidase as described above. In cells expressing a wild-type or LVL/3A mutant core protein, a 23-kDa processed or a 25-kDa unprocessed core protein was detected, as seen in Fig. 3B (Fig. 4D, lanes 2 and 4). The IF176/177AL mutant exhibited a 26-kDa unprocessed band which was detected by anti-HA antibody (Fig. 4D, lane 5). In contrast, the spmt core protein exhibited a major band at 23 kDa and a faint 24-kDa band after blotting with the anti-Flag antibody (Fig. 4D, lane 3). Detection of a small amount of the 24-kDa unprocessed band by the anti-HA antibody indicates that most of the spmt mutant core protein was processed by SPP. The unprocessed core proteins of spmt, LVL/3A and IF176/177AL exhibited different electrophoretic mobilities, estimated to be 24, 25, and 26 kDa, respectively (Fig. 4D, lower panel, lanes 3 to 5). Lemberg and Martoglio pointed out that the mobility of a protein does not necessarily correlate with its molecular mass when analyzed in a Tris-glycine gel system due to the unexpected electrophoretic mobility of the proteins (22). However, detection of HA-tagged unprocessed signal sequence in the core mutants clearly demonstrated that LVL/3A and IF176/177AL mutants substituted with Leu[139], Val[140], and Leu[144] in domain 2 and with Ile[176] and Phe[177] in the transmembrane domain, respectively, have lost the ability to be cleaved by SPP.

Effect of a loss-of-function mutant of SPP on the processing of the signal sequence of HCV core protein. Although there are two reports suggesting that SPP is involved in the processing of the signal sequence of HCV core protein by using the SPP inhibitor $(Z\text{-}LL)_2$-keton (23, 30), a direct interaction of HCV core protein with SPP has not been demonstrated. To determine the direct involvement of SPP in the processing of HCV core protein signal sequence, the C-terminal HA tag in the Flag-Core-E1-HA constructs used in the experiments described above was replaced with a myc/His tag and coexpressed with wild-type SPP (SPP-HAER) or with a mutant SPP with amino acid substitutions in the putative protease active sites, i.e., Asp[219] (SPP D219A-HAER) or Asp[265] (SPP D265A-HAER) to Ala. The signal sequence of HCV core protein was processed in cells coexpressing Flag-Core-E1-myc/His and SPP-HAER (Fig. 5, anti-Flag, lane 3), whereas two bands corresponding to processed and unprocessed (the same size as Flag-Core LVL/3A-E1-myc/His [lane 6]) core proteins were detected in cells coexpressing Flag-Core-E1-myc/His and the mutant SPP constructs (Fig. 5, anti-Flag, lanes 4 and 5). Proper cleavage and glycosylation of E1 proteins in cells coexpressing Flag-Core-E1-myc/His and the SPP mutants (Fig. 5, anti-His, lanes 4 and 5) and those expressing Flag-Core LVL/3A-E1-myc/His (Fig. 5, anti-His, lane 6) indicates that processing of signal sequence by SPP is not required for the cleavage of the core-E1 junction by signal peptidase and translocation of E1 protein into the ER. These results indicate that loss-of-function mutants of SPP inhibit the intramembrane proteolysis of HCV core protein signal sequence and further confirm that the slightly larger bands detected in cells expressing Flag-Core

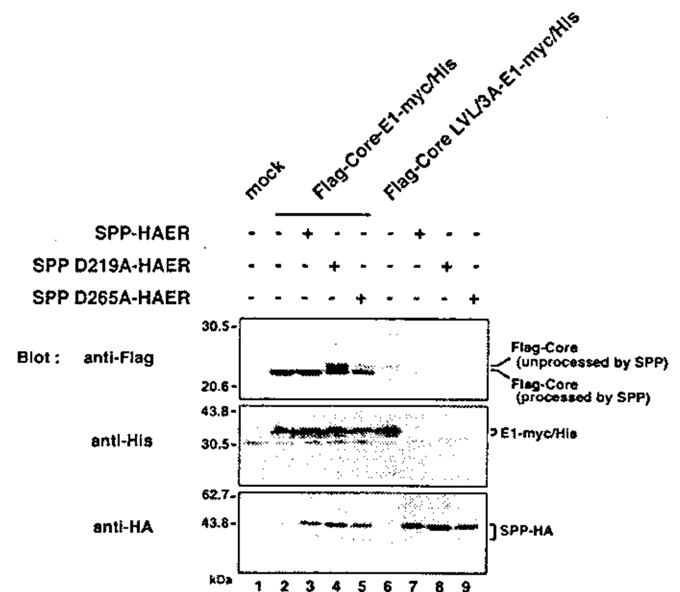

FIG. 5. Effect of loss-of-function mutants of SPP on the processing of the signal sequence of HCV core protein. SPP-HAER, SPPD219A-HAER, or SPPD265A-HAER was coexpressed with Flag-Core-E1-myc/His or Flag-Core LVL/3A-E1-myc/His in 293T cells. Cell lysates were analyzed by immunoblotting with anti-Flag (upper panel), anti-His_6 (middle panel), or anti-HA (lower panel) antibody. + and −, presence or absence of each plasmid, respectively. Lane 1, mock; lanes 2, 6, 7, 8, and 9, single expression of Flag-Core-E1-myc/His, Flag-Core LVL/3A-E1-myc/His, SPP-HAER, SPPD219A-HAER, and SPPD265A-HAER, respectively; lanes 3 to 5, coexpression of Flag-Core-E1-myc/His with SPP-HAER, SPPD219A-HAER, and SPPD265A-HAER, respectively.

LVL/3A-E1-HA or Flag-Core IF176/177AL-E1-HA are immature core proteins unprocessed by SPP (Fig. 4B, lanes 3 and 9).

Interaction of HCV core protein with SPP. To examine the specific interaction of HCV core protein with SPP, Flag-Core-E1-myc/His, Flag-Core LVL/3A-E1-myc/His, or Flag-Core IF176/177AL-E1-myc/His was coexpressed with SPP-HAER or SPP D219A-HAER in 293T cells and immunoprecipitated with anti-Flag or anti-HA antibody. In cells coexpressing the loss-of-function mutant, SPP D219A-HAER, and one of the three HCV polyprotein substrates, nonspecific bands were detected by immunoblotting with the anti-HA and anti-Flag antibodies in the immunoprecipitates (Fig. 6A, upper and second panels, lanes 3 to 5). Therefore, lysates immunoprecipitated with anti-Flag and anti-HA antibodies were evaluated by comparison with those precipitated with anti-EE. Three bands corresponding to SPP D219A-HAER were coimmunoprecipitated with core proteins by anti-Flag immunoprecipitation (Fig. 6A, upper panel, lanes 8 to 10). SPP has two glycosylation sites (50), and therefore the upper, middle, and lower bands seem to correspond to SPP possessing two glycans, one glycan, and no glycan, respectively. Deglycosylation by PNGase F treatment reduced the molecular sizes of all bands to that of the lowest band (data not shown). Only unprocessed core protein was coimmunoprecipitated with SPP D219A-HAER by anti-HA (Fig. 6A, second panel, lanes 8 to 10). Coexpression of Flag-Core LVL/3A-E1-myc/His or Flag-Core-IF176/177AL-E1-myc/His reduced the expression of SPP D219A-HAER (Fig. 6A, third panel, lanes 4 and 5), suggesting that the

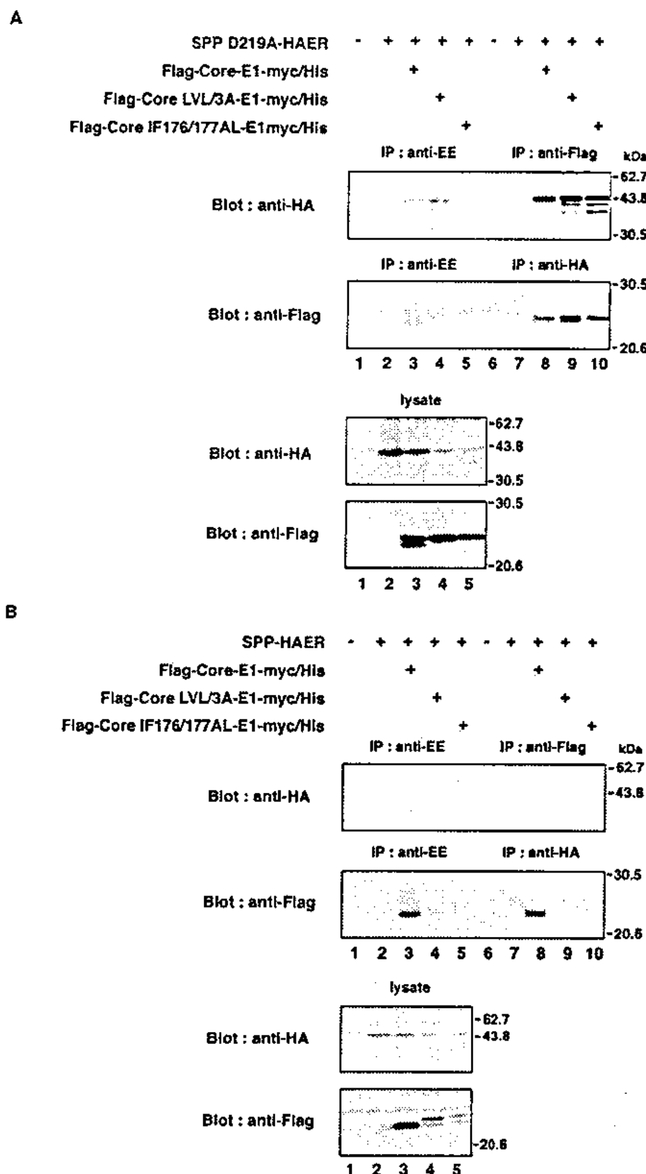

FIG. 6. Interaction of HCV core protein with SPP. SPP D219A-HAER (A) or SPP-HAER (B) was coexpressed with Flag-Core-E1-myc/His, Flag-Core LVL/3A-E1-myc/His, or Flag-Core IF176/177AL-E1-myc/His in 293T cells and immunoprecipitated (IP) with anti-Flag or anti-HA antibody. The immunoprecipitates were analyzed by immunoblotting with anti-HA or anti-Flag antibody. As a control, immunoprecipitation was carried out with anti-EE antibody. + and −, presence or absence of each plasmid, respectively.

core mutants suppress the expression of the SPP mutant. Clear reduction or elimination of the processing of the HCV core protein signal sequence was observed in cells coexpressing SPP D219A-HAER in comparison with those coexpressing wild-type SPP (Fig. 6A and B, bottom panels, lanes 3 to 5). Conversely, no interaction of HCV core protein with wild-type SPP was observed in cells coexpressing SPP-HAER and the HCV polyprotein substrates (Fig. 6B, upper panels, lanes 8 to 10). Broad bands were detected in immunoprecipitates with anti-Flag or anti-EE antibody by immunoblotting with the anti-Flag antibody, probably due to nonspecific binding of the processed

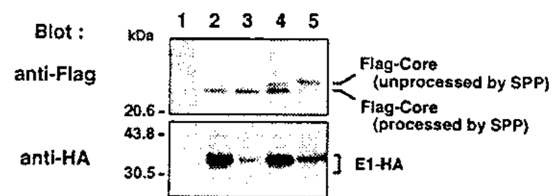

FIG. 7. Processing of HCV core-E1 polyprotein in the human hepatoma cell line FLC-4. Flag-Core-E1-HA (lane 2), Flag-Core ASC/VLV-E1-HA (lane 3), Flag-Core LVL/3A-E1-HA (lane 4), or Flag-Core IF176/177AL-E1-HA (lane 5) was expressed in FLC-4 cells and analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. Cells transfected with an empty plasmid were used as a negative control (lane 1).

core protein to protein G-Sepharose (Fig. 6B, second panel, lanes 3 and 8). These results indicate that a direct interaction of SPP with HCV core protein only between the unprocessed core protein and the loss-of-function mutant of SPP is verifiable. SPP should bind to the signal sequence of HCV core protein and release it after proteolysis, whereas SPP D219A cannot liberate the substrate after binding due to lack of the catalytic activity, suggesting that the SPP mutant may possess dominant negative effects.

Processing of the signal sequence of HCV polyprotein in a human hepatoma cell line. To confirm the data obtained for 293T cells with human liver cells, processing of core-E1 polyprotein in FLC4 cells, a human hepatoma cell line, was examined (Fig. 7). Processing by signal peptidase and SPP was evident in cells expressing Flag-Core-E1-HA or Flag-Core ASC/VLV-E1-HA (lanes 2 and 3), whereas clear processing by signal peptidase, but not complete cleavage by SPP, was observed in FLC-4 cells expressing Flag-Core LVL/3A-E1-HA or Flag-Core IF176/177AL-E1-HA (lanes 4 and 5). These results are consistent with data obtained with 293T cells, suggesting that the processing of the signal sequence of HCV core protein is not cell type dependent or an artifact of the techniques used in this study.

Localization of mutant HCV core proteins. To determine the effect of mutations on the localization of HCV core protein, EGFP-Core 191and its mutants that are defective in cleavage by SPP were expressed in HeLa cells (Fig. 8A). EGFP-Core 191 was processed by SPP and colocalized with an ER marker. EGFP-Core IF176/177AL, which bears a mutation that confers α-helix structure to the signal sequences, was diffusely distributed but did not completely colocalize with ER-DsRed as seen with EGFP-Core 191. EGFP-Core LVL/3A was localized mainly to the nucleus and, to a lesser extent, the cytoplasm, and EGFP-Core Δ128-151 exhibited complete nuclear localization. To confirm the subcellular localization of mutant HCV core proteins, cells were transfected with expression plasmids encoding N-terminally Flag-tagged and C-terminally HA-tagged core proteins to minimize the effect of fusion protein and fractionated, as described in Materials and Methods (Fig. 8B). Consistent with the subcellular localization of EGFP-Core proteins, Flag-Core 191-HA was detected mainly in the membrane-organelle fraction and Flag-Core LVL/3A-HA and Flag-Core Δ128-151-HA were localized mainly in the nuclear fraction. Although EGFP-Core IF176/177AL did not completely colocalize with the ER marker, 55% of Flag-