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A Central Region in the Hepatitis C Virus NS4A Protein Allows Formation of an Active NS3-NS4A Serine Proteinase Complex In Vivo and In Vitro

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A virus-encoded serine proteinase mediates four site-specific cleavages in the hepatitis C virus polyprotein. In addition to the catalytic domain, which is located in the N-terminal one-third of nonstructural protein NS3, the 54-residue NS4A protein is required for cleavage at some but not all sites. Here, we provide evidence for a non-ionic detergent-stable interaction between NS4A and the NS3 serine proteinase domain and demonstrate that the central region of NS4A plays a key role in NS4A-dependent processing. Hydrophobic residues, in particular Ile-29, were shown to be important for NS4A activity, and a synthetic peptide, spanning NS4A residues 22 to 34, could substitute for intact NS4A in a cell-free *trans* cleavage assay. Furthermore, NS4A mutations, which abolished or inhibited processing, correlated with destabilization of the NS3-NS4A complex. These results suggest that a stable interaction exists between the central region of NS4A and the NS3 catalytic domain which is required for NS4A-dependent processing. Since NS4A is required for processing at certain serine proteinase-dependent cleavage sites, this interaction may represent a new target for development of antiviral compounds.

A common feature of positive-strand animal RNA viruses involves the synthesis of polyproteins which are co- and post-translationally cleaved to produce essential viral components of the RNA replication machinery and structural proteins for virion assembly. Such cleavages are usually catalyzed by one or more viral proteinases and, in some cases, host enzymes. This strategy is shared by the hepatitis C viruses (HCV), a group of enveloped, positive-strand RNA viruses, which is now classified as a separate genus in the *Flaviviridae* family, along with the *Flavivirus* and *Pestivirus* genera (reviewed in reference 33). HCV was recently identified as the major causative agent of non-A, non-B posttransfusion hepatitis (6, 21) and is also responsible for a large proportion of community-acquired hepatitis (1). Some HCV infections are asymptomatic, but clinical manifestations can include acute hepatitis, chronic hepatitis, and cirrhosis, which is strongly associated with the development of hepatocellular carcinoma (reviewed in reference 18). Even in immunocompetent individuals chronic infections are common, and current estimates suggest that >1% of the human population has been infected. Protective vaccines are not yet available, and the only approved therapeutic agent, alpha interferon, is effective in <25% of cases (reviewed in reference 18).

HCV genome RNAs are approximately 9.4 kb in length and encode a polyprotein of ~3,000 amino acids (3,011 residues for the HCV H strain [19]). Cell cultures allowing efficient HCV replication have not been established, and our current understanding of HCV polyprotein processing is based on transient expression of cloned HCV cDNAs. For the HCV H strain, the order and nomenclature of the polyprotein cleavage products is NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH, where C, E1, and E2 are putative structural proteins and the remaining nonstructural (NS) proteins are presumed RNA replicase components (11–13, 25) (Fig. 1). Besides host signal peptidase, which appears to catalyze cleavages in the structural-NS2 region (at the C/E1, E1/E2, E2/p7, and p7/NS2 sites) (15, 25, 30, 38), two viral proteinases are responsible for cleavages in the NS region. A novel Zn^{2+}-dependent activity, which encompasses NS2 and the N-terminal ~180 residues of NS3, appears to mediate autocatalytic cleavage at the 2/3 site (12, 17). Distinct from this activity, the N-terminal domain of NS3 also functions as a serine proteinase which cleaves at four downstream sites in the NS region (at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites) (2, 8, 11, 16, 28, 39).

Besides the NS3 serine proteinase domain, which is capable of efficient *trans* cleavage at the 5A/5B site, the 54-residue NS4A protein is required as a "cofactor" for cleavage at the 3/4A and 4B/5A sites (3, 9, 26). The mechanism by which NS4A facilitates cleavage at these sites is not known. In this study, we have used deletion and substitution mutagenesis to map NS4A determinants important for the proteinase cofactor function. Our results define a central region of NS4A, which is shown to be sufficient for its cofactor activity both in vivo and in vitro. In addition, we present evidence for a detergent-stable complex between NS4A and the NS3 serine proteinase domain which is destabilized by NS4A mutations inhibiting NS4A-dependent cleavage. Taken together, these results suggest that the central region of NS4A activates cleavage at NS4A-dependent sites via direct interaction with the NS3 serine proteinase.

MATERIALS AND METHODS

Cell cultures. The BHK-21 cell line was obtained from the American Type Culture Collection. Cell monolayers were grown in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin.

Vaccinia virus-HCV recombinants and expression plasmids. The three vaccinia virus recombinants vTF7-3, vHCV827-3011, and vHCV1027-1207 have been described previously. vTF7-3 expresses the T7 RNA polymerase (10) and is used to transcribe HCV cDNAs located downstream from the T7 promoter and encephalomyocarditis virus leader sequence in recombinant viruses or plasmid

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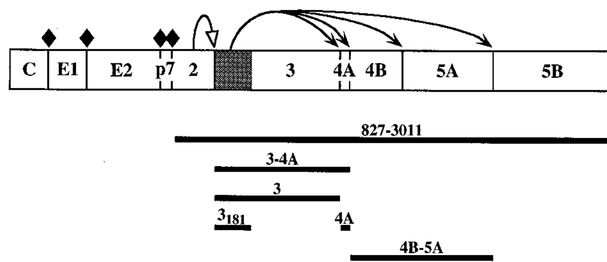

FIG. 1. The HCV polyprotein and expression constructs. The diagram of the HCV H-strain polyprotein indicates the positions and identities of the cleavage products including C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Sites of proteolytic processing are shown at the top, including those for host signal peptidase (◆), the HCV NS2-3 autoproteinase (open arrowhead), and the HCV NS3 serine proteinase (solid arrowheads). The serine proteinase domain in the N-terminal one-third of NS3 is shaded. Shown below are the six expression constructs used in this study. HCV polypeptide sequences present in each plasmid are represented by horizontal lines, which are drawn to scale and oriented with respect to the diagram of the HCV-H polyprotein. For simplicity, the NS prefixes for the nonstructural proteins are not used here or in subsequent figures.

constructs. vHCV827-3011 encodes a polyprotein that begins 17 residues downstream of the NS2 N terminus and extends to the end of the HCV-H open reading frame (13); vHCV1027-1207 encodes the serine proteinase catalytic domain which consists of the N-terminal 181 residues of NS3 ($NS3_{181}$) (26) (Fig. 1).

HCV expression plasmids are identified by numbers specifying the portion of the HCV polyprotein which they encode. For clarity, these designations are followed by the names of the encoded HCV products in parentheses; subscripts refer to the boundaries of truncated proteins. HCV cDNA expression plasmids described previously include pTM3/HCV1027-1207 ($NS3_{181}$), pTM3/HCV1027-1657 (NS3), pBRTM/HCV1027-1711 (NS3-4A), pTM3/HCV1658-1676 ($NS4A_{1-19}$), pTM3/HCV1658-1692 ($NS4A_{1-35}$), pTM3/HCV1658-1711 (NS4A), and pTM3/HCV1712-2420 (NS4B-5A) (26) (Fig. 1).

Standard recombinant DNA techniques were used for construction of the following plasmids (36). Ten constructs encoding NS4A derivatives with substitution mutations were generated from pTM3/HCV1658-1711 (NS4A) by site-directed mutagenesis (20, 24). Mutations were identified by creating new restriction enzyme sites and confirmed by dideoxynucleotide sequence analysis. Encoded NS4A mutants are described by their position in the NS4A sequence. For instance, NS4A mutant V_4L_5>AA contains Ala substitutions at both the fourth (Val) and the fifth (Leu) residues of NS4A. Restriction enzyme recognition sites and the corresponding mutations were *Pst*I for V_4L_5>AA, V_9L_{10}>AA, L_{13}>A, $S_{19}T_{20}$>AA, $V_{23}V_{24}I_{25}$>AAV, $I_{25}V_{26}$>AA, $I_{29}V_{30}L_{31}$>AAV, and $L_{31}S_{32}$>AA; *Bss*HII for $C_{22}V_{23}$>AL, G_{27}>A, $R_{28}I_{29}$>AL, and K_{34}>A; *Bgl*II for I_{29}>S; and *Eco*RV for V_{30}>S.

In addition to pTM3/HCV1658-1676 ($NS4A_{1-19}$) and pTM3/HCV1658-1692 ($NS4A_{1-35}$), 10 plasmids containing various N- or C-terminal NS4A deletions were created by using the pTM3/HCV1658-1711 (NS4A) mutants just described. Four C-terminal deletion constructs, pTM3/HCV1658-1679 ($NS4A_{1-22}$), pTM3/HCV1658-1681 ($NS4A_{1-24}$), pTM3/HCV1658-1685 ($NS4A_{1-28}$), and pTM3/HCV1658-1687 ($NS4A_{1-30}$), were generated by deleting a *Pst*I-*Nhe*I fragment from pTM3/HCV1658-1711 (NS4A) containing the mutations $V_{23}V_{24}I_{25}$>AAV, $I_{25}V_{26}$>AA, $I_{29}V_{30}L_{31}$>AAV, and $L_{31}S_{32}$>AA, respectively. The *Pst*I- and *Nhe*I-digested termini were trimmed and filled in, respectively, by T4 DNA polymerase prior to ligation. Six plasmids with N-terminal deletions, pTM3/HCV 1663-1711 ($NS4A_{6-54}$), pTM3/HCV1668-1711 ($NS4A_{11-54}$), pTM3/HCV1672-1711 ($NS4A_{15-54}$), pTM3/HCV1678-1711 ($NS4A_{21-54}$), pTM3/HCV1683-1711 ($NS4A_{26-54}$), and pTM3/HCV1684-1711 ($NS4A_{27-54}$), were generated by deleting an *Nco*I-*Pst*I fragment from pTM3/HCV1658-1711 (NS4A) containing the mutations V_4L_5>AA, V_9L_{10}>AA, L_{13}>A, $S_{19}T_{20}$>AA, $V_{23}V_{24}I_{25}$>AAV, and $I_{25}V_{26}$>AA, respectively. Before ligation, the *Nco*I- and *Pst*I-digested termini were filled in and trimmed, respectively, by T4 DNA polymerase. pTM3/HCV1693-1711 ($NS4A_{36-54}$) was created by deletion of an *Nco*I-*Nae*I fragment from pTM3/HCV1658-1711 (NS4A), in which the *Nco*I cohesive end was filled in by T4 DNA polymerase prior to ligation.

Three constructs with both N- and C-terminal deletions were generated by using the NS4A deletion constructs. First, mutation $L_{31}S_{32}$>AA or K_{34}>A was introduced into pTM3/HCV1678-1711 ($NS4A_{21-54}$) by site-directed mutagenesis. pTM3/HCV1678-1687 ($NS4A_{21-30}$) was then derived from the $L_{31}S_{32}$>AA mutant by deleting the region between the *Pst*I and *Nhe*I sites, with both termini trimmed and filled in, respectively, by T4 DNA polymerase prior to ligation. pTM3/HCV1678-1690 ($NS4A_{21-33}$) was generated from the K_{34}>A mutant by deleting a *Bss*HII-*Nhe*I fragment, in which both termini were filled in by T4 DNA polymerase before ligation. pTM3/HCV1678-1692 ($NS4A_{21-35}$) was produced from pTM3/HCV1678-1711 ($NS4A_{21-54}$) by deleting an *Nco*I-*Nae*I fragment and filling in the *Nco*I cohesive end by T4 DNA polymerase prior to ligation.

Transient expression using the vaccinia virus-T7 hybrid system. For expression assays utilizing the vaccinia virus-HCV recombinants, monolayers of BHK-21 cells in 35-mm-diameter dishes were infected with vTF7-3 alone or in combination with vHCV827-3011 or vHCV1027-1207. The multiplicity of infection for each recombinant was 10 PFU per cell. After adsorption for 60 min at room temperature, the inoculum was removed and replaced with MEM containing 2% FBS. Expression assays of transfected plasmids utilized subconfluent monolayers of BHK-21 cells, which had been previously infected with vTF7-3 as described above. Some of them were coinfected with vHCV1027-1207 as indicated. After removal of the inoculum, cells were transfected for 2 h at 37°C with 1 μg of plasmid DNA premixed with 15 μg of Lipofectamine (GIBCO BRL) in 0.5 ml of MEM. For cotransfection experiments with two plasmids, the amount of DNA was 0.5 μg for each construct.

For *trans* cleavage experiments, monolayers were washed once with prewarmed methionine-deficient MEM at 3 h postinfection and then incubated for 4 h at 37°C with MEM containing 1/40 of the normal concentration of methionine and cysteine, 2% FBS, and 40 μCi of a ^{35}S protein labeling mixture (New England Nuclear) per ml. For coimmunoprecipitation experiments, cell monolayers were incubated with MEM containing 2% FBS for 1 h at 37°C after transfection. Then, the cell monolayers were washed once with prewarmed MEM deficient in both methionine and cysteine and incubated for 2 h at 37°C with the same type of medium containing 2% FBS and 40 μCi of both [^{35}S]methionine and [^{35}S]cysteine per ml. Finally, the labeling media were replaced with MEM containing 2% FBS, and the cells were incubated for 1 h at 37°C.

Cell lysis, immunoprecipitation, and protein analyses. (i) Denaturing conditions. ^{35}S-labeled cell monolayers were washed with phosphate-buffered saline and lysed in 0.3 ml of denaturing buffer containing 0.5% sodium dodecyl sulfate (SDS), 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, and 20 μg of phenylmethylsulfonyl fluoride per ml. Cellular DNA was sheared by repeated passage through a 27.5-gauge needle. Cell lysates were heated for 10 min at 70°C, and 100 μl of each lysate was diluted with 3 volumes of TNA (35) and incubated with an HCV-specific antiserum. Immune complexes were collected by using *Staphylococcus aureus* Cowan I (Calbiochem) (35), solubilized, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (22) or Tricine-SDS-PAGE (37). After treatment for fluorography with En^3Hance (Dupont), gels were dried and exposed at −70°C with prefogged (23) X-ray film (Kodak). ^{14}C-methylated molecular weight marker proteins were purchased from Amersham.

(ii) Nondenaturing conditions. For coimmunoprecipitation experiments, ^{35}S-labeled cell monolayers were washed and lysed in 0.6 ml of nondenaturing buffer A containing 0.5% Triton X-100, 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, and 150 mM NaCl, supplemented with proteinase inhibitors aprotinin (10 μg/ml), benzamidine (1 mM), E-64 (10 μM), leupeptin (100 μM), and phenylmethylsulfonyl fluoride (60 μg/ml). Nuclei and other cell debris were removed by centrifugation at 16,000 × *g* for 10 min at 4°C. Two hundred microliters of the clarified lysates was incubated overnight with 5 μl of a rabbit polyclonal antiserum (WU43, WU110, or WU117) (13) or 2 μl of the M5 monoclonal antibody, which is specific for residues 1697 to 1708 of the HCV polyprotein (NS4A residues 40 to 51) (a gift from S. Devare). Immune complexes were collected with protein A-agarose (Sigma) and washed six times with buffer A supplemented with 60 μg of phenylmethylsulfonyl fluoride per ml. The beads were then washed once with buffer A without Triton X-100, solubilized, and analyzed by SDS-PAGE or Tricine-SDS-PAGE.

Cell-free proteinase assay. Since pTM3-derived HCV expression constructs contain an upstream T7 promoter followed by the internal ribosome entry site of encephalomyocarditis virus, 5′-uncapped RNA transcripts were synthesized from linearized cDNA templates by using T7 RNA polymerase (Epicenter) (34). Cell-free translations using rabbit reticulocyte lysates (Promega) were performed according to the manufacturer's instructions. To produce ^{35}S-labeled NS4B-5A substrate, transcripts from pTM3/HCV1712-2420 (NS4B-5A) were incubated for 60 min at 30°C in the presence of 300 μCi of [^{35}S]methionine (Amersham) per ml. Unlabeled proteinase components were translated by using transcripts from pTM3/HCV1027-1207 ($NS3_{181}$) and pTM3/HCV1658-1711 (NS4A) for 90 min at 30°C with a mixture containing all 20 unlabeled amino acids (Promega). In some cases, microsomal membranes (Promega) were added to a final concentration of 1.8 μl/25-μl reaction. Translation reactions were terminated by the addition of RNase A (Boehringer Mannheim) to 10 μg/ml and cycloheximide to 0.3 mg/ml, and the mixtures were incubated for 10 min at 30°C. Unlabeled methionine (to 1 mM) was also added to the ^{35}S labeling reaction mixtures.

After termination of the translation reactions, *trans* cleavage reactions were initiated by mixing proteinase and substrate products in a 1:1 ratio and incubating the mixtures at 30°C for 3 h (27). A synthetic peptide, H-Cys-Val-Val-Ile-Val-Gly-Arg-Val-Leu-Ser-Gly-Lys-OH, corresponding to residues 22 to 34 of NS4A, was supplied by Bachem Bioscience, Inc. Reversed-phase high-pressure liquid chromatography and mass-spectral analysis indicated that the peptide purity was >96%. Stock solutions of the peptide (1.5 mM) were prepared in 0.2 M acetic acid. Sequential dilutions of the peptide were made in 20 mM acetic acid and assayed by adding 1/4th volume to the cleavage reactions prior to the 3-h incubation. Equivalent additions of carrier solvent showed no effect on NS3 *trans* cleavage activity. After incubation, the cleavage reaction mixtures were diluted 10-fold with Laemmli sample buffer, heated for 5 min at 95°C, and analyzed by SDS-PAGE.

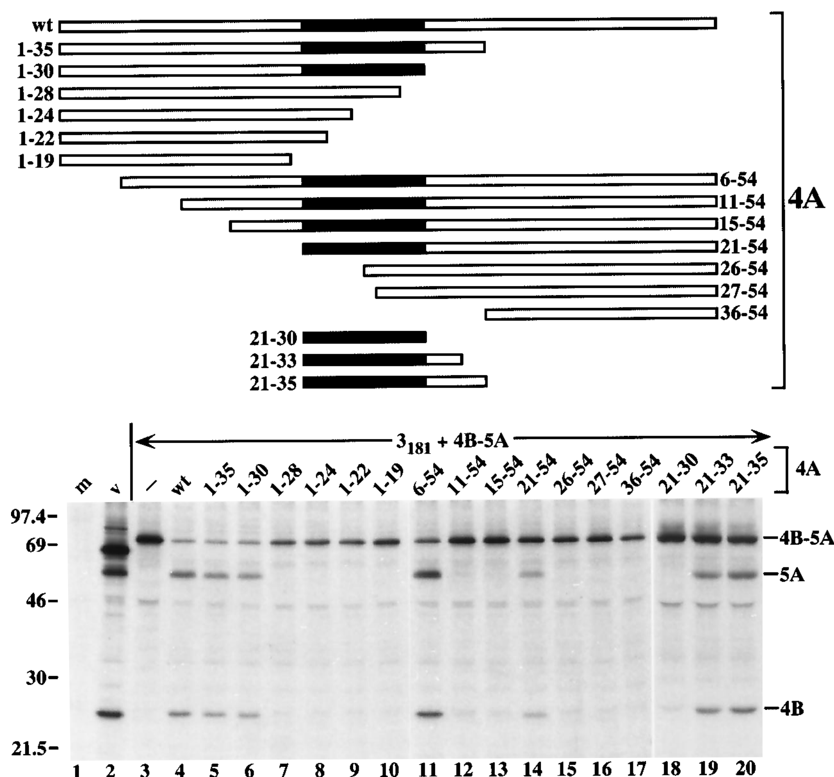

FIG. 2. Analysis of NS4A deletion mutants. BHK-21 cell monolayers were infected with vTF7-3 alone (m) or in combination with vHCV827-3011 (v) or vHCV1027-1207 (3_{181}). Some monolayers were also transfected with pTM3/HCV1712-2420 (4B-5A) or the wild-type NS4A expression construct pTM3/HCV1658-1711 (wt) or its derivatives expressing the deletion mutants indicated above the lanes. Cells were labeled with a ^{35}S protein labeling mixture as described in Materials and Methods. HCV-specific products were immunoprecipitated by using human patient serum JHF, solubilized, and separated by SDS–10% PAGE. Deleted forms of NS4A were not detected with this antiserum (data not shown). In a parallel experiment, similar results were obtained by using an independent clone for each NS4A mutant construct. In this and subsequent figures, HCV-specific proteins are indicated on the right, and the sizes of ^{14}C-labeled protein molecular weight markers (in kilodaltons) are indicated on the left. At the top, the diagram shows NS4A and the various deletion mutants. As mapped by this deletion analysis, the central region of NS4A implicated in cofactor function is shown as a solid bar (this is not meant to imply that this region alone is sufficient for cofactor activity).

RESULTS

The central region of NS4A is essential for proteinase cofactor function. C-terminal deletion analysis of the NS3-4A polyprotein, which functions efficiently for *trans* cleavage of the NS4B-5A substrate, suggested that NS4A sequences downstream of residue 35 were dispensable for cofactor activity (26). To further define NS4A determinants important for its role in NS3 serine proteinase-dependent cleavage at the 4B/5A site, we used the vaccinia virus-T7 hybrid system to coexpress the serine proteinase domain ($NS3_{181}$), the NS4B-5A substrate, and full-length or various deletion derivatives of NS4A in BHK-21 cells. As shown previously, $NS3_{181}$ is able to process the NS4B-5A substrate only in the presence of coexpressed NS4A (26) (see Fig. 2, lanes 3 and 4). Truncated NS4A constructs with C-terminal deletions up to NS4A residue 30 ($NS4A_{1-35}$ and $NS4A_{1-30}$) were able to activate $NS3_{181}$-mediated *trans* cleavage of NS4B-5A (Fig. 2, lanes 5 and 6), whereas deletion of an additional sequence ($NS4A_{1-28}$, $NS4A_{1-24}$, $NS4A_{1-22}$, or $NS4A_{1-19}$) abolished detectable proteolytic activity (lanes 7 to 10). Removal of the N-terminal five amino acids of NS4A ($NS4A_{6-54}$) did not significantly affect the efficiency of cleavage at the 4B/5A site (lane 11). However, N-terminal deletions to residue 11 or 15 ($NS4A_{11-54}$ or $NS4A_{15-54}$) eliminated processing (lanes 12 and 13). Interestingly, further truncation to residue 21 ($NS4A_{21-54}$) restored NS4A cofactor activity, although NS4B-5A cleavage appeared to be somewhat less efficient (lane 14). Deletion of additional N-terminal residues ($NS4A_{26-54}$, $NS4A_{27-54}$, or $NS4A_{36-54}$) abolished detectable processing (lanes 15 to 17). These results suggest that the central region of NS4A, between residues 21 and 30, is essential for its cofactor function.

Further support for this conclusion was obtained by expression of shorter polypeptides encompassing the central region of NS4A. Both $NS4A_{21-33}$ and $NS4A_{21-35}$ enabled the $NS3_{181}$ proteinase to process NS4B-5A (lanes 19 and 20). In contrast, the peptide corresponding to the minimal region mapped by deletion analysis, $NS4A_{21-30}$, was not active (lane 18). Such negative results are difficult to interpret but could result from poor translation, instability, improper folding, or other factors which might affect cofactor activity, such as subcellular localization. Alternatively, these data may indicate that additional upstream or downstream NS4A sequences may be necessary for full cofactor activity.

Importance of multiple hydrophobic residues in the central region of NS4A. By inspection, the 54-residue NS4A polypeptide can be divided into three regions: an N-terminal one-third rich in hydrophobic amino acids; a central region consisting of hydrophobic, hydrophilic, and charged residues; and a C-terminal portion that is highly charged, containing 7 acidic residues (Fig. 3). As just described, the studies using deleted forms of NS4A suggested a key role for the central region of NS4A in cofactor activity, although residues in the N-terminal region may also participate. To identify the specific residue(s) which might be important for NS4A cofactor function, a limited num-

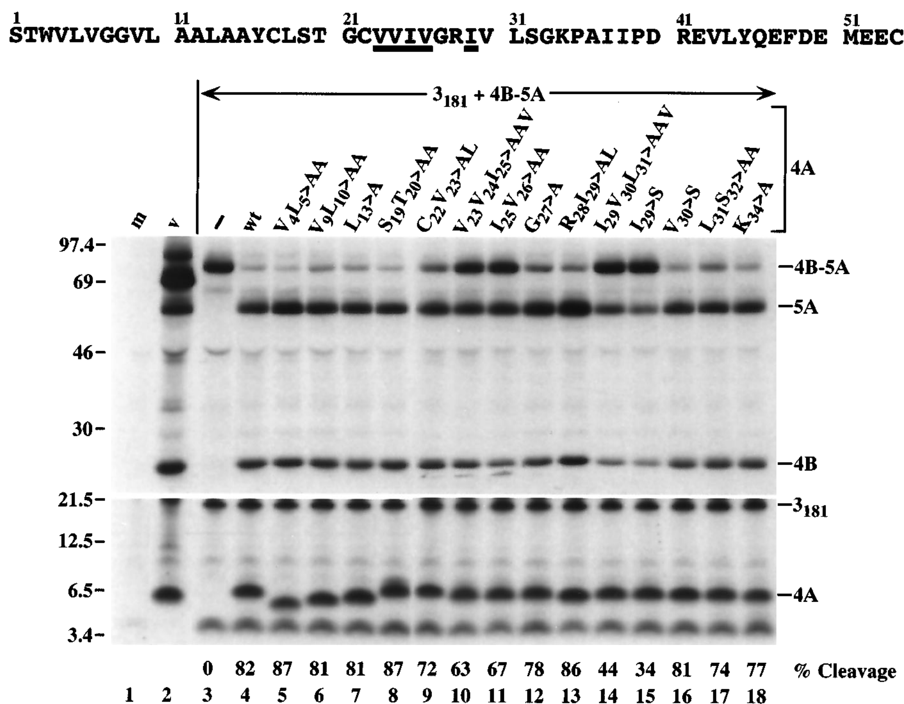

FIG. 3. Analysis of NS4A substitution mutants. The NS4A sequence for the HCV H strain, numbered from the N-terminal residue of NS4A (11), is shown at the top. The central region of NS4A is shaded, and hydrophobic residues particularly important for cofactor activity are underlined. Fourteen NS4A substitution mutants (indicated above the lanes) coexpressed with the $NS3_{181}$ proteinase, were assayed for *trans* cleavage activity by using the NS4B-5A substrate. HCV-specific proteins were immunoprecipitated by using a combination of JHF and WU110 antisera and analyzed by SDS–10% PAGE (upper gel) or Tricine–SDS–14% PAGE (lower gel). NS4A accumulation for each of the mutants was similar to the wild-type level. Several NS4A substitution mutations appeared to affect the migration of the protein in Tricine-SDS-PAGE. Although only one set of data is presented, similar results were obtained in a parallel experiment using independent clones for each NS4A mutant construct. The percent cleavage {100 × [products/(precursor + products)]}, as determined by densitometry, is given below each lane. Experimental details and figure labels are similar to those for Fig. 2.

ber of multiple and single substitution mutations were examined by the same expression assay. As expected from the deletion analysis, multiple Ala substitutions in the N-terminal region of NS4A showed little effect on the efficiency of NS4B-5A processing by the $NS3_{181}$ proteinase (Fig. 3, lanes 5 to 8). This was also true for three mutations in the central region (G_{27}>A, $R_{28}I_{29}$>AL, and K_{34}>A) (lanes 12, 13, and 18). Slight inhibition was observed for two other substitutions in the central region ($C_{22}V_{23}$>AL and $L_{31}S_{32}$>AA; lanes 9 and 17). More dramatic inhibition was seen with Ala substitutions for Val or Ile residues in the central region ($V_{23}V_{24}I_{25}$>AAV, $I_{25}V_{26}$>AA, and $I_{29}V_{30}L_{31}$>AAV; lanes 10, 11, and 14). The $I_{29}V_{30}L_{31}$>AAV mutant was least efficient for NS4B-5A processing, whereas only a slight defect was observed for the $L_{31}S_{32}$>AA mutant (compare lanes 14 and 17), suggesting that either Ile-29, Val-30, or both residues might be important for NS4A cofactor activity. To test the importance of these residues, individual substitutions were created at each of these positions. Ser, a hydrophilic amino acid, was chosen as a less conservative amino acid replacement, given the rather subtle effects seen with the Ala substitutions. Substitution of Ser for Ile-29 but not Val-30 caused a significant loss of cofactor activity (compare lanes 15 and 16). These data suggest an important role for multiple hydrophobic residues in the NS4A central region, especially Ile-29, in cofactor activity.

Evidence for a detergent-stable complex between NS4A and the NS3 serine proteinase domain. The requirement for NS4A as a serine proteinase cofactor and the observation that cotranslation of HCV NS4A facilitates localization of NS3 to microsomal membranes (17) suggest that these two proteins may interact to form a complex which might be the active proteinase responsible for cleavage at NS4A-dependent sites. To address this possibility, various forms of NS3 and NS4A (Fig. 4A) were coexpressed by using the vaccinia virus-T7 system, and complex formation was assayed by coimmunoprecipitation under nondenaturing conditions. For the NS3-4A polyprotein, cleavage at the 3/4A site occurs rapidly in *cis* (3, 26) and no uncleaved NS3-4A polyprotein was detected under our labeling conditions (Fig. 4B and data not shown). For cell lysates prepared with Triton X-100, both NS3 and NS4A were coprecipitated when an antiserum directed against the NS3 helicase domain (Fig. 4B, lane 2) or a monoclonal antibody specific for the C-terminal region of NS4A (lane 3) was used. Only a very faint NS4A band was detected with an antiserum directed against the NS3 serine proteinase domain (lane 1). Although less efficient, coprecipitation was also observed when full-length NS3 and NS4A were coexpressed from separate plasmids (Fig. 4B, lanes 4 to 6). These immunoprecipitation conditions appeared to be specific since NS3 expressed alone was not recognized by the M5 antibody (lane 9) nor was NS4A recognized by the NS3-specific antisera (lanes 10 and 11). Although this approach does not allow firm conclusions concerning a direct association between NS3 and NS4A to be drawn, the data provide evidence for a detergent-stable complex containing these two proteins. As mentioned earlier, antiserum against the NS3 helicase domain (WU117) coprecipitated NS4A much more efficiently than the WU43 antiserum recognizing the serine proteinase domain. This observation suggests a direct interaction between NS4A and the NS3 serine proteinase domain. The WU43 antiserum might disrupt this

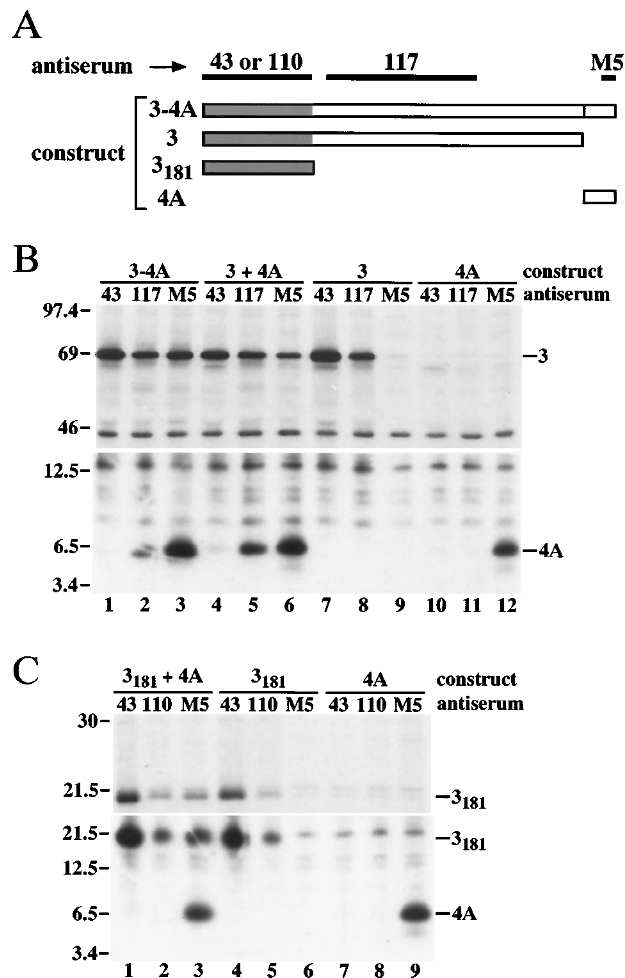

FIG. 4. Evidence for NS3-NS4A and $NS3_{181}$-NS4A complexes. (A) Schematic of the expressed HCV polypeptides and the region-specific antisera used for immunoprecipitation. (B) vTF7-3-infected BHK-21 cell monolayers were transfected with plasmid pBRTM/HCV1027-1711 (3-4A), pTM3/HCV1027-1657 (3), or pTM3/HCV1658-1711 (4A) or cotransfected with both pTM3/HCV1027-1657 and pTM3/HCV1658-1711 (3 + 4A). (C) vTF7-3-infected BHK-21 cell monolayers were transfected with pTM3/HCV1027-1207 (3_{181}) or pTM3/HCV1658-1711 (4A) or cotransfected with both pTM3/HCV1027-1207 and pTM3/HCV1658-1711 (3_{181} + 4A). As described in Materials and Methods, cells were labeled with a ^{35}S protein labeling mixture, and HCV-specific products were immunoprecipitated under nondenaturing conditions by using the antibodies indicated above the lanes. WU43 and WU110 antisera (43 and 110 in the figure) react with the NS3 proteinase domain, WU117 (117 in the figure) reacts with the NS3 helicase domain, and M5 is a monoclonal antibody which recognizes the C-terminal portion of NS4A. Immunoprecipitates were solubilized and separated by SDS–8% PAGE (upper gel in panel B), SDS–12% PAGE (upper gel in panel C), or Tricine–SDS–16% PAGE (lower gels in panels B and C).

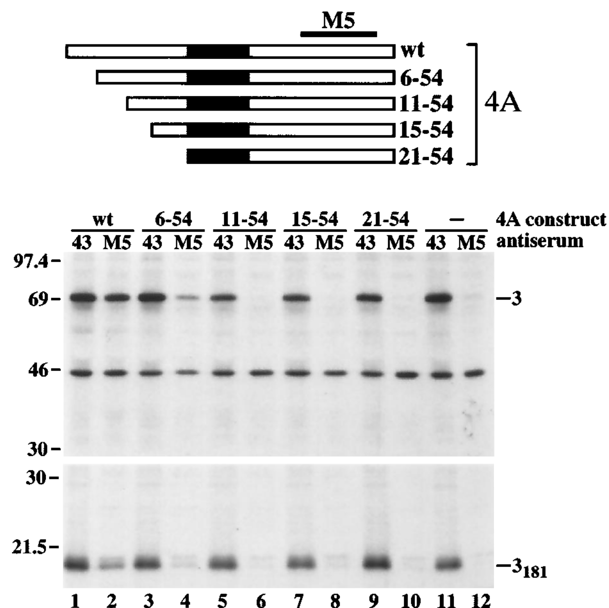

FIG. 5. Effects of NS4A deletion mutations on formation of detergent-stable complexes with NS3 or $NS3_{181}$. vTF7-3-infected BHK-21 cell monolayers were transfected with pTM3/HCV1027-1657 (3) (upper gel) or pTM3/HCV1027-1207 (3_{181}) (lower gel). Some monolayers were cotransfected with the wild-type NS4A expression construct pTM3/HCV1658-1711 (wt) or derivatives expressing the N-terminally deleted forms indicated above the lanes (and diagrammed above as in Fig. 2). Cells were labeled, and HCV-specific products were immunoprecipitated as described for Fig. 4. Immunoprecipitated products were analyzed by SDS–8% PAGE (upper gel) or SDS–12% PAGE (lower gel).

interaction, or, alternatively, NS4A could mask NS3 epitopes recognized by this antiserum, leading to inefficient immunoprecipitation of the complex. In contrast, the M5 monoclonal antibody, which recognizes NS4A residues 40 to 51, could efficiently coprecipitate NS3. These data indicate that the C-terminal portion of NS4A, besides being dispensable for cofactor activity, is probably also not directly involved in complex formation.

Further support for an interaction between the NS3 serine proteinase domain and NS4A was obtained by coexpression of $NS3_{181}$ and NS4A. As shown in Fig. 4C, $NS3_{181}$ could be specifically coprecipitated by the NS4A-specific M5 antibody (lane 3). As before, NS4A was not coprecipitated by either of two independent antisera raised against the serine proteinase domain (lanes 1 and 2).

NS4A mutations deleterious for cofactor activity destabilize NS3-NS4A complexes. We next examined whether there was a correlation between NS4A cofactor activity and our ability to recover NS3-NS4A complexes. Several of the N-terminal NS4A deletion mutants, which would be expected to react with the NS4A-specific M5 monoclonal antibody, were tested by cotransfection with either full-length NS3 or $NS3_{181}$. As shown in Fig. 5, for $NS4A_{6-54}$ only a small fraction of NS3 was coprecipitated (lane 4) compared with the wild-type NS4A (lane 2). No coprecipitation with NS3 was observed for $NS4A_{11-54}$ (lane 6), $NS4A_{15-54}$ (lane 8), or $NS4A_{21-54}$ (lane 10). Although complicated by a nonspecific background doublet migrating in the vicinity of $NS3_{181}$ (Fig. 4C), similar results were obtained for $NS3_{181}$ (Fig. 5, lower gel).

We also tested several of the NS4A derivatives with substitutions in the central region. As assayed by the level of coprecipitated NS3 or NS4A (Fig. 6), two single substitution mutations (V_{30}>S and K_{34}>A) had no detectable effect on recovery of NS3-NS4A complexes. Substitutions in five mutants, including $C_{22}V_{23}$>AL, $I_{25}V_{26}$>AA, G_{27}>A, $R_{28}I_{29}$>AL, and $L_{31}S_{32}$>AA, appeared to destabilize the NS3-NS4A complexes to various degrees but not completely disrupt them. For the other three mutants, $V_{23}V_{24}I_{25}$>AAV, $I_{29}V_{30}L_{31}$>AAV, and I_{29}>S, no detectable NS4A was coprecipitated by the NS3-specific helicase antibody, and the amount of NS3 precipitated by the M5 monoclonal antibody was near the nonspecific background level observed in some experiments (Fig. 4B, lane 9).

Together with the results presented earlier, these experiments show that deletion or substitution mutations, which abolish or inhibit cofactor activity, either block the formation

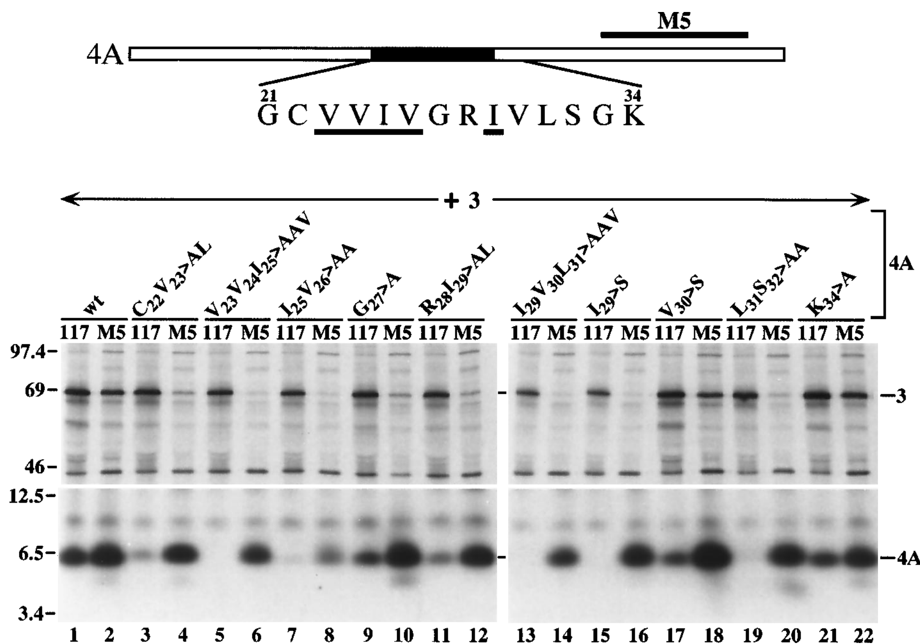

FIG. 6. Effects of NS4A substitution mutations on formation of detergent-stable complexes. The diagram of NS4A shown at the top indicates the central region implicated in cofactor function (solid bar), its sequence from residue 21 to 34, and the region of NS4A recognized by the M5 monoclonal antibody. For this analysis, vTF7-3-infected BHK-21 cell monolayers were cotransfected with pTM3/HCV1027-1657 (3 in the figure) and the wild-type NS4A expression construct pTM3/HCV1658-1711 (wt) or its derivatives expressing mutated NS4A proteins with the indicated substitutions. Cells were labeled, and HCV-specific products were immunoprecipitated as described for Fig. 4. Samples were analyzed by SDS–8% PAGE (upper gels) or Tricine–SDS–14% PAGE (lower gels).

of NS3-NS4A complexes or destabilize them, such that they are no longer resistant to the conditions used for nondenaturing immunoprecipitation. These results are consistent with the hypothesis that cleavages at NS4A-dependent sites are mediated by a proteinase complex consisting of NS3 and NS4A. However, the ability to recover complexes was not strictly correlated with cofactor activity, since several of the mutations led to diminished recovery of NS3-NS4A complexes but had no observable effect in the *trans* cleavage assay. These included the N-terminal deletion mutant NS4A$_{6-54}$ and substitution mutants G$_{27}$>A and R$_{28}$I$_{29}$>AL. One possibility is that these mutations may still allow the formation of active proteinase complexes in vivo but result in weaker NS3-NS4A interactions such that complexes are disrupted by the conditions used for nondenaturing coprecipitation (see Discussion).

A synthetic peptide encompassing the central region of NS4A possesses proteinase cofactor activity in vitro. Our analyses of NS4A deletion and substitution mutants in vivo suggested that the central region of NS4A might be sufficient for cofactor activity. Using a recently developed cell-free *trans* cleavage assay (27), we tested the activity of a synthetic peptide corresponding to NS4A residues 22 to 34. In this assay, *trans* cleavage was monitored after unlabeled proteinase components and radiolabeled substrates produced by cell-free translation were mixed. Radiolabeled NS4B-5A was not processed by the NS3$_{181}$ proteinase alone (27) (data not shown), but, as evidenced by the appearance of NS4B, site-specific cleavage occurred upon addition of both NS3$_{181}$ and NS4A (Fig. 7, lane 2). As shown previously (27), the efficiency of *trans* cleavage improved when NS4A was translated in the presence of microsomal membranes (lane 7). When the NS4A synthetic peptide was substituted for NS4A, dose-dependent processing of the NS4B-5A substrate was observed. Peptide concentrations of 30 and 10 μM led to more efficient processing than authentic NS4A translated in the absence of microsomal membranes (compare lanes 2 to 4). Detectable processing was observed even in the presence of 3 μM peptide (lane 5). Similar results were obtained when the peptide was added to NS3$_{181}$ translated in the presence of microsomal membranes. However, under these conditions, the highest concentration of peptide tested (30 μM; lane 8) was not as efficient as authentic NS4A translated in the presence of microsomal membranes (lane 7)

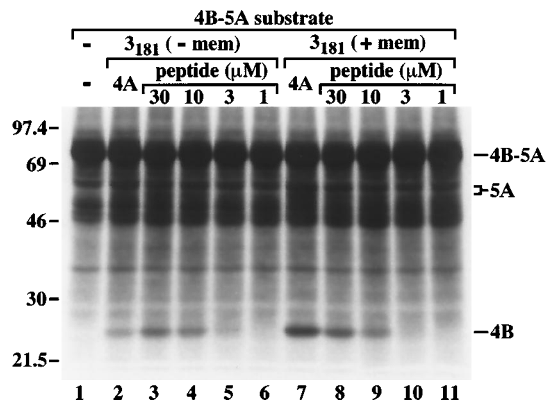

FIG. 7. In vitro activity of an NS4A synthetic peptide. Cell-free transcription and translation were used to synthesize the indicated HCV proteins. The ^{35}S-labeled NS4B-5A substrate was synthesized in the absence of microsomal membranes (lanes 1 to 11). Unlabeled proteinase components were produced by translation in the absence (− mem) (lanes 2 to 6) or presence (+ mem) (lanes 7 to 11) of microsomal membranes. In lanes 2 and 7, NS3$_{181}$- and NS4A-encoding RNAs were translated in the same reaction; in lanes 3 to 6 and 8 to 11, NS3$_{181}$-encoding RNA was translated alone. For *trans* cleavage assays, translation reactions containing the substrate or the proteinase components were mixed in a 1:1 ratio and incubated for 3 h at 30°C. As indicated, a synthetic peptide corresponding to NS4A residues 22 to 34 was added to a final concentration of 30 μM (lanes 3 and 8), 10 μM (lanes 4 and 9), 3 μM (lanes 5 and 10), or 1 μM (lanes 6 and 11). Products were analyzed by SDS–10% PAGE.

and NS4A synthetic peptide activity did not appear to be stimulated by microsomal membranes. The ability of the NS4A synthetic peptide to function as a proteinase cofactor in vitro appears to be specific since NS4A peptides of similar length containing nonconservative substitutions appear to be inactive, even at high peptide concentrations (14).

DISCUSSION

In addition to the HCV NS3 serine proteinase domain, the NS4A protein is required as a cofactor for efficient processing at the 3/4A and 4B/5A polyprotein cleavage sites (3, 9, 26). Our experiments, which focused on *trans* cleavage of an NS4B-5A substrate, suggest that an NS3-NS4A complex may be the active form of the serine proteinase responsible for cleavage at NS4A-dependent sites. In contrast, NS4A is not required for efficient *trans* cleavage at the 5A/5B site (3, 26), although coexpression of NS4A has been reported to enhance processing at this site (9).

Our data demonstrate that the central region of NS4A is important for its cofactor function. Analysis of deletion mutants in vivo implicated the NS4A coding region corresponding to residues 21 to 30 as essential for cofactor function. Further, a peptide consisting of NS4A residues 22 to 34 was shown to be functional for cofactor activity in a cell-free *trans* cleavage assay. The latter experiment formally proves that this region of the NS4A sequence acts as a cofactor at the level of the encoded polypeptide rather than as an RNA element and further maps the essential region to residues 22 to 30. The sequence of this region of HCV-H NS4A is Cys-22–Val–Val–Ile–Val–Gly–Arg–Ile–Val-30, and substitution mutations at some, but not all, of these residues inhibited cofactor activity. Of the mutations examined, Ala substitutions for the bulkier hydrophobic residues (Val-23 to Val-26 and Ile-29) significantly inhibited NS4A activity whereas little or no effect was seen for Cys-22, Gly-27, or Arg-28. The most dramatic inhibition was observed for the more hydrophilic Ser substitution at Ile-29. The normal activity seen for the $R_{28}I_{29}$>AL mutant and in vitro activity of the NS4A synthetic peptide, which contained a Val at position 29, suggest that hydrophobic character is important at this position. In agreement with these observations, Val is found at this position in another genotype 1a isolate, HCV-1 and Leu is found at this position for more distantly related genotype 2 subtypes HCV-J6 and HCV-J8 (see reference 26 for sequence alignments). Although additional analyses are needed, these data suggest that multiple interactions involving hydrophobic residues in the central region of NS4A, especially residue 29, are important for cofactor function.

The linkage between formation of an NS3-NS4A complex and activation of the proteinase for cleavage at NS4A-dependent sites is less clear. Mutations in the central region which inhibited cofactor activity led to decreased recovery of detergent-stable NS3-NS4A complexes. However, deletions in the N-terminal portion of NS4A or substitutions in the central domain which did not affect activity also led to diminished recovery of NS3-NS4A complexes. While not absolutely required for complex formation and cofactor activity in vivo, certain residues in the N-terminal and central regions of NS4A may interact with the NS3 serine proteinase domain and contribute to the stability of the NS3-NS4A complex and its resistance to disruption by the conditions used for nondenaturing coprecipitation (in particular, inclusion of the detergent Triton X-100). The fact that this complex is destabilized by a number of different mutations suggests that the overall association between NS4A and NS3 may be weak and may involve multiple interactions.

The mechanism(s) by which NS4A enables the NS3 serine proteinase to cleave at the 4B/5A site is still unclear. One possibility is that NS4A might act as a chaperone to mediate proper folding of the serine proteinase. Although NS4A may participate in NS3 folding or contribute to its stability, two lines of evidence argue against an obligate chaperone-like role for NS4A. First, the NS3 serine proteinase domain expressed in the absence of NS4A is active and can cleave at the 5A/5B site efficiently in vivo (3, 26) or inefficiently in vitro (27). Second, NS3 proteinase produced by cell-free translation can be activated for cleavage of the NS4B-5A substrate by posttranslational addition of the synthetic NS4A peptide or the NS4A translation product (this study) (27). NS4A might also function by anchoring the active NS3 proteinase to cellular membranes where polyprotein substrates containing the hydrophobic NS4 region are likely to be localized (17). Although membrane localization is likely to be important for proteinase function during normal processing in cells (see below), the cell-free *trans* cleavage experiments show that membranes are not absolutely required for NS4A cofactor activity (Fig. 7) (27). Rather, we favor a model in which the central region of NS4A interacts with the NS3 serine proteinase domain to alter cleavage site specificity and allow processing at NS4A-dependent sites. Although biochemical and structural studies are needed to refine our understanding of NS4A action, several nonexclusive mechanisms can be envisioned. For instance, NS4A might bind to the serine proteinase and change the conformation of the substrate binding pocket to allow recognition and cleavage of NS4A-dependent sites (without interfering with recognition of the 5A/5B site). Alternatively, NS4A bound to the proteinase domain could contribute directly to substrate recognition through interactions with NS4A-dependent substrates.

As shown for other positive-strand RNA viruses (see reference 7 for a review), *cis* versus *trans* polyprotein processing events and the different substrate specificities of the HCV NS3 and NS3-NS4A proteinases could play an important regulatory role in viral replication. Pulse-chase analyses (3, 26) and dilution experiments (3) suggest that HCV 2/3 and 3/4A cleavages are rapid and occur in *cis*. The other three cleavages by the serine proteinase (4A/4B, 4B/5A, and 5A/5B) can occur in *trans* (3, 9, 26), but it is unknown if they can also occur in *cis*. Since NS3 and NS4A may form a complex after *cis* cleavage at the 3/4A site and NS4A facilitates membrane association of NS3 (17), two forms of the serine proteinase—a membrane-bound NS3-NS4A complex and "free" NS3 in the cytosol, which may have different *trans* cleavage activities—may exist in HCV-infected cells. The ratio of these proteinases should change during the course of HCV replication, favoring the formation of the complex as viral proteins accumulate. Changes in the concentration and/or specificity of these *trans*-acting proteinases could modulate polyprotein processing and thereby affect the composition and function of RNA replication complexes. However, elucidation of such regulatory pathways will require the development of methods for studying the HCV RNA replication machinery.

Like the HCV NS3-NS4A serine proteinase complex, multicomponent proteinases with catalytic and regulatory subunits are not uncommon among cellular and viral enzymes. Members of the *Flavivirus* genus utilize a complex consisting of NS2B and NS3 for processing at all serine proteinase-dependent sites (reviewed in reference 33). The pestiviruses may also encode a cofactor modulating the activity of the p80 serine proteinase (41). Other examples include the adenovirus proteinase, which is activated by an 11-residue virus-derived peptide (29, 40) and the cowpea mosaic virus 24K proteinase, whose activity is modulated by a second virus-encoded protein (32).

For HCV, the NS3 serine proteinase and the NS4A cofactor are required for efficient processing at two or perhaps three sites in the viral polyprotein (3, 9, 26). By analogy to the flaviviruses (4, 5, 31) and other positive-strand RNA viruses, these cleavages are likely to be essential for HCV replication. Although the HCV NS2-3 autoproteinase and the NS3 serine proteinase are being pursued as targets for development of therapeutic compounds, it is likely that successful control or eradication of chronic HCV infections will require multiple antiviral agents with distinct modes of action. The interaction between NS4A and the NS3 catalytic domain provides another such target.

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