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Complex Formation between the NS3 Serine-Type Proteinase of the Hepatitis C Virus and NS4A and Its Importance for Polyprotein Maturation

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Processing of the hepatitis C virus polyprotein is mediated by host cell signalases and at least two virally encoded proteinases. Of these, the serine-type proteinase encompassing the amino-terminal one-third of NS3 is responsible for cleavage at the four sites carboxy terminal of NS3. The activity of this proteinase is modulated by NS4A, a 54-amino-acid polyprotein cleavage product essential for processing at the NS3/4A, NS4A/4B, and NS4B/5A sites and enhancing cleavage efficiency between NS5A and NS5B. Using the vaccinia virus-T7 hybrid system to express hepatitis C virus polypeptides in BHK-21 cells, we studied the role of NS4A in proteinase activation. We found that the NS3 proteinase and NS4A form a stable complex when expressed as a single polyprotein or as separate molecules. Results from deletion mapping show that the minimal NS4A domain required for proteinase activation is located in the center of NS4A between amino acids 1675 and 1686 of the polyprotein. Amino acid substitutions within this domain destabilizing the NS3-NS4A complex also impair *trans* cleavage at the NS4A-dependent sites. Similarly, deletion of amino-terminal NS3 sequences impairs complex formation as well as cleavage at the NS4B/5A site but not at the NS4A-independent NS5A/5B site. These results suggest that a stable NS3-NS4A interaction is important for cleavage at the NS4A-dependent sites and that amino-terminal NS3 sequences and the central NS4A domain are directly involved in complex formation.

Hepatitis C viruses (HCV) were identified as the main agents of the parentally transmitted form of non-A, non-B hepatitis (for a recent review, see reference 7). HCV cause the vast majority of transfusion-associated cases of hepatitis and account for a significant proportion of community-acquired hepatitis cases worldwide. Most, if not all, infections with HCV become chronic and result in various clinical outcomes including acute hepatitis, chronic hepatitis, liver cirrhosis, or the establishment of an asymptomatic carrier state which may persist for life. Furthermore, the increased incidence of hepatocellular carcinoma in non-A, non-B hepatitis patients suggests an important role of HCV in the process of hepatocarcinogenesis (7, 35).

Sequence comparisons between various HCV cDNAs isolated during the past few years indicate the existence of several genotypes which can diverge by as much as 50% at the amino acid level (reference 36 and references cited therein). They are now grouped as the separate genus hepatitis C virus together with the two other genera *Flavivirus* and *Pestivirus* in the family *Flaviviridae*. These viruses have in common a virion with a lipid envelope and a single-stranded nonsegmented RNA of positive polarity ranging in size between 9.4 (HCV) and 12.5 (pestivirus) kb. The RNA contains a single long open reading frame which in the case of HCV is expressed as a polyprotein of about 3,010 amino acids and which is flanked by 5′ and 3′ nontranslated regions. Given the lack of a convenient tissue culture system to propagate HCV, cell-free translation and cell culture expression studies were employed to analyze polyprotein maturation. At least 10 cleavage products are generated from the polyprotein precursor and they are arranged in the order NH_2-core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (18, 19, 21, 26, 30, 34, 39). Core, a basic RNA-binding protein, is assumed to be the major constituent of the viral nucleocapsid (reference 32 and references cited therein), E1 and E2 are most likely virion envelope glycoproteins (reference 9 and references cited therein), and NS2 to NS5B are nonstructural proteins (most of them probably involved in RNA replication). Host cell signal peptidase located in the lumen of the endoplasmic reticulum is responsible for maturation of the structural proteins (19, 26, 30). At least two virally encoded proteinases are involved in processing of the NS proteins. A zinc-dependent metalloproteinase encompassing the NS2 region and the amino-terminal one-third of NS3 (the NS2-3 proteinase) is required for cleavage between NS2 and NS3 (16, 20, 22). Processing at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites is mediated by a serine-type proteinase which is also encoded in the amino-terminal one-third of NS3 but functioning independently from the NS2-3 proteinase (4, 10, 17, 20, 29, 39). We and others have recently shown that NS4A is an important NS3 serine-type proteinase cofactor required for cleavage at the NS3/4A, NS4A/4B, and NS4B/5A sites and enhancing cleavage efficiency between NS5A and NS5B (5, 12, 27, 38).

The requirement for a proteinase cofactor is reminiscent of the situation of the closely related flaviviruses such as yellow fever virus and dengue virus. Processing of their NS proteins and maturation of nucleocapsid protein are mediated essentially by a proteinase heterodimer composed of the NS3 proteinase and the NS2B cofactor (reference 1 and references cited therein). Since mutations in NS2B affecting complex formation also impair proteolytic processing by NS3, formation of

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a stable physical interaction between NS2B and NS3 appears to be an essential prerequisite for NS polyprotein maturation (2, 6).

In this study we tested the possibility of complex formation between the HCV NS3 proteinase and the NS4A cofactor. Using the vaccinia virus-T7 hybrid system, we could demonstrate a stable interaction between the NS3 proteinase domain and NS4A. Destabilization of the complex by NS3 or NS4A deletions or amino acid substitutions reduced cleavage efficiency at the NS3/4A, NS4A/4B, and NS4B/5A sites, suggesting that NS3-NS4A interaction is an essential prerequisite for proteinase activation.

MATERIALS AND METHODS

Cells and viruses. Cell monolayers of the BHK-21 cell line were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U of penicillin, 100 μg of streptomycin, and 10% fetal calf serum. The recombinant vaccinia virus vTF7-3 expressing the RNA polymerase of bacteriophage T7 (15) was obtained from B. Moss (National Institutes of Health, Bethesda, Md.). Stocks of recombinant vaccinia viruses were grown in human TK^- 143 cell monolayers, and titers of infectious progeny were determined by plaque assay with the same cell line.

Plasmid constructions. Standard recombinant DNA techniques were used for construction of the expression plasmids (31). Synthetic oligonucleotides were used to introduce initiation and termination codons by PCR methodology, using Vent polymerase according to the instructions of the manufacturer (New England Biolabs, Schwalbach/Taunus, Germany) and a program of 15 cycles. In all cases, a 5′ *Nco*I site and a 3′ *Spe*I site were engineered for insertion into pTM1-2. Transferred PCR fragments were verified by DNA sequence analysis. For all plasmids, the numbers refer to the first and last amino acids of the expressed HCV protein (Fig. 1).

To simplify the insertion of HCV fragments, the multiple cloning site of the basic vector pTM1 (11; provided by B. Moss) was modified by restriction with *Sal*I, filling in the 5′ protruding ends by using the Klenow enzyme (New England Biolabs), and religation. The resulting vector pTM1-1 was digested with *Bam*HI, the 5′ protruding ends were filled in, and the plasmid was cut with *Stu*I and religated. The resulting vector pTM1-2, in which the *Pst*I, *Xho*I, and *Stu*I restriction sites of the multiple cloning site were deleted while the *Bam*HI site was restored, was used for all subsequent cloning steps. For tagging with the hemagglutinin (HA) epitope, two complementary oligonucleotides were inserted into the *Nco*I-*Spe*I-digested pTM1-2. All HCV fragments introduced into the unique *Nco*I site of this vector (pHA) downstream of the epitope sequence were expressed as fusion proteins carrying at their amino termini the sequence **MYPY DVPDYA**PM (bold letters indicate the HA epitope).

The basic plasmid pTM1007-3011 directing the expression of a polyprotein from NS3 (plus 20 amino acids derived from the carboxy terminus of NS2; Fig. 1) up to the stop codon of NS5B was obtained by insertion of an *Nco*I-*Sal*I fragment and a *Sal*I-*Spe*I fragment derived from pATA1007-3011 (5) into pTM1-2. To obtain plasmid pTM1007-3011/3A, directing the expression of the analogous polyprotein but with a substitution of the proteinase active-site serine residue by an alanine, an *Nco*I-*Sal*I fragment and a *Sal*I-*Spe*I fragment, derived from pATA1007-3011/S→A (5), were inserted into pTM1-2 via *Nco*I and *Spe*I. pTM809-3011 was constructed by insertion of an *Nco*I-*Sal*I fragment isolated from pATA809-3011 and a *Sal*I-*Spe*I fragment isolated from a pATA derivative containing the complete HCV open reading frame plus 76 nucleotides of the 5′ nontranslated region into pTM1-2. Construction of pTM1007-3011/4P was done by insertion of a *Sal*I-*Bsp*EI PCR fragment, carrying the NS4A substitution, and a *Bsp*EI-*Spe*I fragment derived from pTM1007-3011 into pTM1007-3011, which had been digested with *Sal*I and *Spe*I. Plasmid pTM1712-3011 was derived from pTM1007-3011 by substitution of the 3,351-bp *Nco*I-*Eco*RI fragment by the 1,225-bp *Nco*I-*Eco*RI PCR fragment. pTM1007-1711 was derived from pTM1007-3011 by substituting the 4,083-bp *Nsi*I-*Spe*I fragment by the 188-bp *Nsi*I-*Spe*I PCR fragment. pTM1659-1711 was obtained by insertion of the 162-bp *Nco*I-*Spe*I PCR fragment into pTM1-2. Plasmids carrying various 5′-terminal NS3 fragments (pTM1027-1238, pTM1034-1238, pTM1041-1238, pTM1049-1238, and pTM1065-1238) were obtained by insertion of *Nco*I-*Xba*I PCR fragments into the *Nco*I-*Spe*I-digested pTM1-2. Plasmids directing the expression of various NS4A fragments carrying at their amino termini the HA epitope (pHA1659-1711, pHA1659-1692, pHA1659-1688, pHA1659-1686, pHA1659-1679, pHA1675-1711, and pHA1683-1711) were obtained by insertion of *Nco*I-*Spe*I PCR fragments into the *Nco*I-*Spe*I-digested pHA. Mutations of NS4A were generated by PCR by using synthetic oligonucleotides introducing the desired nucleotide exchange and carrying the *Bsp*EI site of our isolate. The resulting 93-bp *Nco*I-*Bsp*EI PCR fragment was inserted into the *Nco*I-*Bsp*EI-digested pTM1659-1711.

Antisera. Antisera directed against NS3 (amino acids 1007 to 1246), NS3 and NS4 (named 3/4; amino acids 1616 to 1738), NS5A (amino acids 2101 to 2231), and NS5B (amino acids 2419 to 2622) have been described (4, 5). The NS4A-specific antiserum was obtained by immunization of rabbits with a MalE-NS4A fusion protein (amino acids 1658 to 1711 of the HCV polyprotein). Antiserum directed against NS4A and NS4B (amino acids 1618 to 1960; kindly provided by L. Tomei [Instituto di Richerche di Biologia Molecolare, Rome, Italy]) has been described previously (12). The antiserum directed against the HA epitope was purchased from Hiss Diagnostics (Freiburg, Germany).

Transient expression with the vaccinia virus-T7 hybrid system. A total of 3.5×10^5 cells seeded in 35-mm-diameter dishes 24 h before the experiment were infected with vTF7-3 at a multiplicity of infection of 5 to 10 for 1 h at room temperature. After removal of the inoculum, cells were incubated in DMEM containing fetal calf serum and glutamine for 30 min at 37°C and then transfected by using 3 μg of purified plasmid DNA and 10 μl of lipofectamine (Life Technologies, Eggenstein, Germany) corresponding to 20 μg of liposomes in a total volume of 1 ml of Optimem medium (Life Technologies) according to the instructions of the manufacturer. When cells were transfected with two or three different plasmids, the amount of each plasmid was reduced to 1.5 μg. After 3 h at 37°C, cells were washed with prewarmed DMEM lacking methionine and incubated for 4 h in DMEM supplemented with 2% dialyzed fetal calf serum, 2 mM glutamine, 2.5 μM methionine, and protein labeling mixture (50 μCi/ml) (Translabel; ICN Biomedicals, Meckenheim, Germany).

Cell lysis, immunoprecipitation, and protein gel electrophoresis. For immunoprecipitation under nondenaturing conditions, cell monolayers were washed with phosphate-buffered saline and lysed with NPB (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with 1 mM phenylmethylsulfonyl fluoride and 0.001 trypsin inhibitor unit per ml of aprotinin (Sigma, Deisenhofen, Germany). Cell lysates were cleared by centrifugation at $13,800 \times g$ for 15 min at 4°C, and portions of each lysate were used for immunoprecipitation with polyclonal rabbit antisera preadsorbed to protein A-Sepharose (Sigma) as described recently (5). Immunoprecipitations under denaturing conditions were performed as previously described (4). For the experiment shown in Fig. 9, samples were prepared in the same way but with a modified NPB buffer containing the same components but with only 0.5% sodium deoxycholate and 0.05% SDS. The best separation of NS4A was obtained by Tricine-SDS-polyacrylamide gel electrophoresis (PAGE). Because using this gel system, we could not obtain sufficient separation of HCV proteins with higher molecular weights from background in most cases, these proteins were analyzed by SDS-PAGE. Gels were treated for fluorography with sodium salicylate and exposed at −70°C with Biomax X-ray films (Sigma).

RESULTS

Complex formation between NS3 and NS4A. Numerous studies have shown that the amino-terminal domain of NS3 encodes a serine-type proteinase required for cleavage at all four sites carboxy terminal of NS3 (Fig. 1). Although enzymatically active on its own, the 54-amino-acid NS4A is required as a cofactor to modulate proteinase activity (5, 12, 27, 38). Cleavages at the NS3/4A, NS4A/4B, and NS4B/5A sites strictly depend on NS4A, whereas processing between NS5A and NS5B also occurs in its absence, but at least for some substrates, cleavage efficiency at this site is increased by NS4A. Although NS3 proteinase modulation is now well documented, so far the mechanism by which NS4A exerts this effect is not known.

By making an analogy to flavivirus NS2B/3 proteinase, we assumed that modulation of the HCV NS3 proteinase might be mediated by interaction with NS4A. To test this hypothesis experimentally, we used the vaccinia virus-T7 hybrid system to examine the influence of NS4A on cleavage of an NS4B-5B substrate by the NS3 proteinase. This enzyme was expressed as a 211-amino-acid amino-terminal NS3 fragment sufficient for full proteolytic activity (5) ($NS3_{211}$ [Fig. 1]). The NS4B-5B substrate was chosen because it allows direct comparison of cleavage efficiencies at an NS4A-dependent cleavage site and an NS4A-independent cleavage site (the NS4B/5A site and the NS5A/5B site, respectively). Complex formation was monitored by coimmunoprecipitation of NS3 and NS4A from the lysate of transfected cells.

As shown in Fig. 2A, uncleaved NS4B-5B substrate was detected in cells transfected with pTM1712-3011 (lanes 1 to 4). This protein was precipitated with antiserum directed against the 20 carboxy-terminal NS3 amino acids, NS4A, and the amino-terminal half of NS4B (anti-3/4 [lane 2]) as well as with antisera monospecific for NS5A (lane 3) and NS5B (lane 4) but

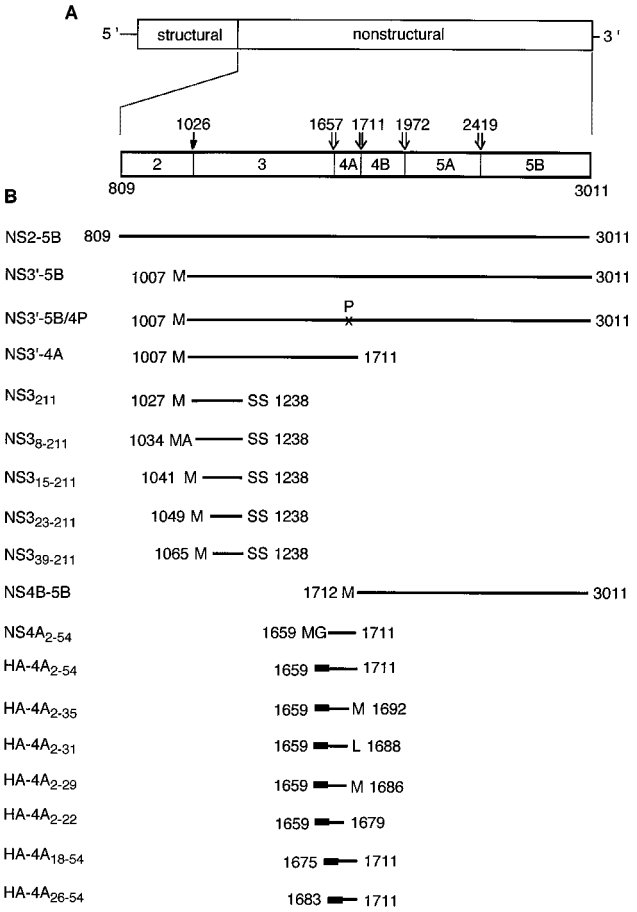

FIG. 1. HCV genome structure and expression constructs. (A) A schematic representation of the HCV polyprotein with the structural proteins encoded in the amino-terminal quarter followed by the nonstructural proteins is shown on top. The 5′ and 3′ nontranslated regions are indicated by the thin black lines. A detailed view of the nonstructural proteins NS2 to NS5B (2 to 5B in figure) (amino acids 809 to 3011 of the polyprotein of our isolate) including the cleavage sites for the NS2-3 proteinase (↓) and the NS3 proteinase (⇓) is drawn below. The numbers above the arrows refer to amino acids at the P1 positions of the scissile bonds. (B) Summary of the HCV expression constructs used in this study. The numbers at the sides of the lines refer to the first and last amino acids of the polyprotein of our isolate. Amino acids at the amino or carboxy termini of expressed proteins derived from the multiple cloning site are given in single-letter code. The polypeptide designation is given to the left of the corresponding expression construct. Subscript numbers refer to the first and last amino acids of the particular NS protein. The HA epitope tag is indicated as a black box (for sequence, see Materials and Methods).

not with the antiserum directed against the NS3 proteinase domain (lane 1). Mature NS5B was generated from this polyprotein in cells cotransfected with the proteinase domain (lane 8), whereas NS4B and NS5A were found exclusively as uncleaved NS4B-5A intermediate together with significant amounts of uncleaved precursor (lane 7). As shown by the production of mature NS5A, cleavage at the NS4B/5A junction was found in cells expressing the proteinase domain and NS4A (lane 11), demonstrating the essential role of NS4A for processing at this site (we were not able to detect NS4B under these conditions, but detection of NS5A was taken to indicate cleavage at the NS4B/5A junction). Furthermore, the absence of uncleaved precursor also indicates an increased cleavage efficiency between NS5A and NS5B.

A first hint as to how NS4A might modulate the NS3 proteinase was given by the coprecipitation shown in Fig. 2A, lanes 9 and 10. When NS3 was isolated by immunoprecipitation under nondenaturing conditions using the NS3-specific antiserum, both NS3 and NS4A were detected, although NS4A alone was not precipitated with this antiserum (see below). On the other hand, NS3 was always coprecipitated with NS4A by using the NS3/4-specific antiserum which did not directly react with the NS3 proteinase domain (compare lane 6 with lane 10 in Fig. 2A), demonstrating formation of a detergent-stable complex.

To analyze whether this complex also forms when NS3 and NS4A were expressed as a single NS polyprotein precursor and to identify possible interactions of NS3 or NS4A with other NS proteins, cells were transfected with a construct directing the expression of an NS2-5B polyprotein (pTM809-3011). To differentiate between coprecipitation and possible cross-reactivity, HCV-specific proteins were isolated from half of the cell lysate by immunoprecipitation under nondenaturing conditions and from the other half after denaturation. As shown in Fig. 2B, both NS4A and the NS4AB processing intermediate were coprecipitated under native conditions with NS3 by using the NS3-specific antiserum (lane 6), whereas this complex was destroyed when samples were denatured prior to immunoprecipitation (lane 1). Conversely, NS3 was coprecipitated with the NS4A-specific antiserum (lane 8) but was not detected by immunoprecipitation of the denatured sample (lane 3). These results demonstrate that the NS3 proteinase can form a complex both with NS4A and with the NS4AB intermediate, showing that cleavage at the NS4A/4B site is not essential for this interaction. Apart from the NS3-NS4A complex, no other coprecipitation was found under these conditions.

To determine the stability of the NS3/4A complex, a pulse-chase experiment was performed with an NS3′-4A proteinase (pTM1007-1711). As shown in Fig. 3, the relative proportions of the two proteins did not change over time. Steady-state labeling of cells transfected with the same construct yielded similar results (data not shown), demonstrating that NS3 and NS4A form a stable complex. Complex formation was also detected when NS4A was expressed as part of an NS4A-5B substrate and released by *trans* cleavage at the NS4A/4B site (data not shown). Furthermore, coprecipitation was found after translation of this construct in a rabbit reticulocyte lysate or after expression in *Escherichia coli* (data not shown). Enzymatic activity was not required for interaction, because NS4A could be efficiently coprecipitated with an inactive NS3 mutant in which the active-site serine residue was substituted by an alanine (data not shown).

Structural and functional characterizations of NS4A. To map the minimal NS4A domain required for interaction with NS3 and proteinase activation, a series of NS4A proteins containing amino- and carboxy-terminal deletions were constructed and tested for the activation function and coprecipitation. In the initial set of experiments, we found that most of these truncated proteins did not react with our NS4A-specific antiserum. Therefore, they were fused at their amino termini with the HA epitope (Fig. 1 and Materials and Methods) and isolated by immunoprecipitation under nondenaturing conditions with a polyclonal rabbit antiserum specifically recognizing the HA portion. Cells were transfected with constructs directing the expression of the NS4B-5B substrate (pTM1712-3011), the NS3 proteinase domain (pTM1027-1238), and one of the HA-4A constructs. As controls, cells expressing only the substrate and proteinase (Fig. 4, lanes 1 and 2) or the substrate and NS4A (lanes 3 and 4) were analyzed in parallel. Proteinase activation was monitored by comparing the relative proportions of the NS4B-5A intermediate and NS5A. Given the different expression levels, protein stabilities, and immunoreac-

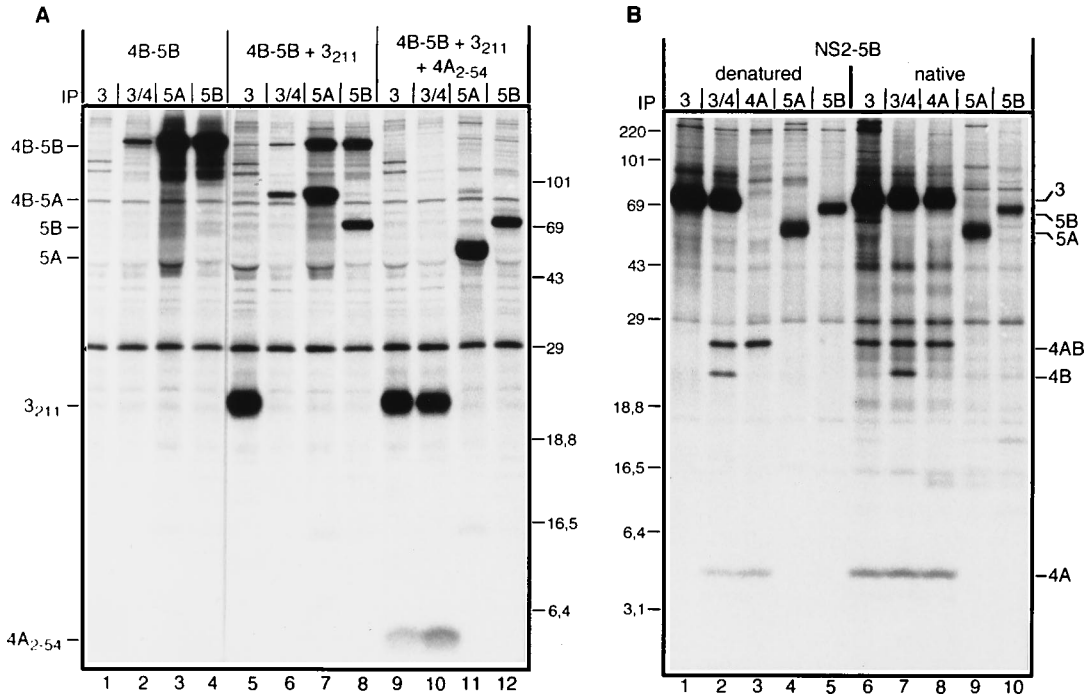

FIG. 2. Complex formation between the NS3 proteinase and NS4A. (A) BHK-21 cell monolayers previously infected with the vTF7-3 recombinant vaccinia virus were transfected with pTM1712-3011 (lanes 1 to 4), with pTM1712-3011 and pTM1027-1238 (lanes 5 to 8), or with pTM1712-3011, pTM1027-1238, and pTM1659-1711 (lanes 9 to 12) as described in Materials and Methods. Three hours after transfection, proteins were radiolabeled metabolically with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 4 h. HCV-specific proteins were isolated from the cell lysate by immunoprecipitation (IP) with the antiserum given above each lane under nondenaturing conditions. Immunocomplexes were solubilized and separated by electrophoresis on a Tricine–11% polyacrylamide gel. HCV-specific proteins are identified on the left, the positions of marker proteins (in kilodaltons) are given on the right. (B) vTF7-3-infected cells were transfected with the construct directing the expression of the NS2-5B polyprotein, and HCV-specific proteins were isolated by immunoprecipitation under nondenaturing conditions (lanes 6 to 10) or after denaturation (lanes 1 to 5). HCV-specific proteins and the positions of protein molecular mass standards (in kilodaltons) are given on the right and left, respectively.

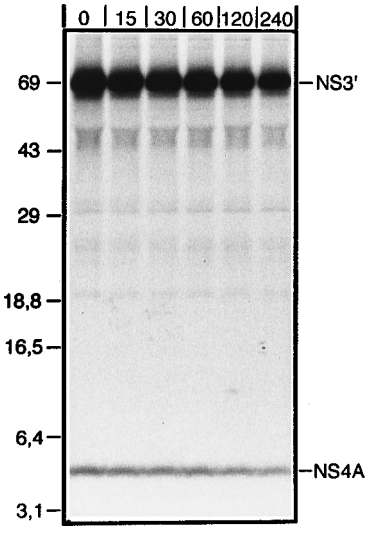

FIG. 3. Stability of the NS3/4A complex. BHK-21 cells infected with vTF7-3 were transfected with the construct directing the expression of the NS3′-4A proteinase (pTM1007-1711). Three hours after transfection, cells were labeled for 15 min and lysed either directly or after incubation in nonradioactive medium for the indicated times (in minutes). Immunoprecipitations with the NS3-specific antiserum were done under nondenaturing conditions. For further details, see the legend to Fig. 2.

tivities of the proteins with various NS4A truncations, attempts to quantify activation efficiencies were not undertaken. Efficient coprecipitation and proteinase activation were found for the parental NS4A fused with the HA epitope (HA-4A$_{2–54}$; Fig. 4, lanes 5 and 6). No difference between this parental NS4A and the HA-4A fusion protein lacking 19 residues at the carboxy terminus of NS4A (HA-4A$_{2–35}$; lanes 7 and 8) was observed. Further deletion of four amino acids (HA-4A$_{2–31}$) strongly impaired the NS3-NS4A interaction (Fig. 4A, lanes 9 and 10) and reduced overall cleavage efficiency, as seen by increased amounts of uncleaved NS4B-5B substrate and the NS4B-5A intermediate and reduced amounts of mature NS5A (Fig. 4B, lanes 9 and 10). Removal of 25 carboxy-terminal residues (HA-4A$_{2–29}$) reduced coprecipitation below the detection limit and significantly impaired proteinase activation (Fig. 4, lanes 11 and 12). Deletion of further seven amino acids abolished cleavage at the NS4B/5A site within the limits of detection (HA-4A$_{2–22}$; Fig. 4B, lanes 13 and 14). Thus, the gradual loss of proteinase activation observed for these NS4A truncations was paralleled by a gradual reduction of coprecipitation, indicating the importance of NS3-NS4A interaction for proteinase activation. Deletions at the NS4A amino terminus severely reduced protein expression, stability, or immunoreactivity because none of these proteins could be detected with the HA epitope-specific antiserum. Strongly reduced amounts of NS5A and no coprecipitation of NS3$_{211}$ were observed for the HA-4A fusion protein lacking 17 amino-terminal residues (HA-4A$_{18–54}$; lanes 15 and 16), whereas no proteinase activation was found when 25 amino acids were removed (HA-4A$_{26–54}$; lanes

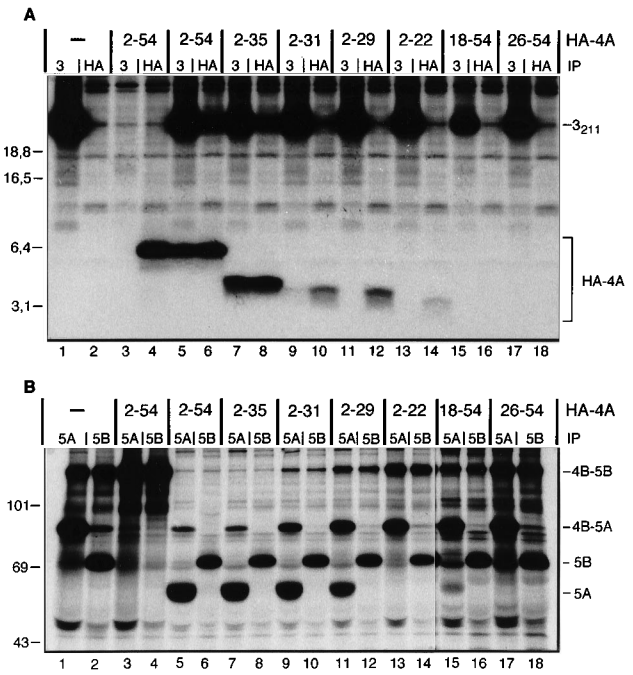

FIG. 4. Mapping of the minimal NS4A domain required for NS3 proteinase activation. Cells previously infected with vTF7-3 were transfected with pTM1712-3011 (NS4B-5B) and pTM1027-1238 ($NS3_{211}$; lanes 1 and 2 in panels A and B), with pTM1712-3011 and pHA1659-1711 (HA-$4A_{2-54}$; lanes 3 and 4), or with pTM1712-3011, pTM1027-1238, and one of the HA-4A constructs specified above the lanes. Proteins were radiolabeled metabolically, and HCV-specific proteins were isolated by immunoprecipitation (IP). Proteins precipitated with the NS3-specific or HA epitope-specific antiserum were separated by electrophoresis on Tricine–13% polyacrylamide gels (A) and proteins precipitated with the NS5A- or NS5B-specific antiserum were separated by electrophoresis on SDS–11% polyacrylamide gels (B). Identification of HCV proteins is given on the right. Numbers on the left refer to the sizes of marker proteins (in kilodaltons). To demonstrate the small amounts of NS5A in the transfection with pHA1675-1711 (HA-$4A_{18-54}$) and for comparison with the result of the HA-$4A_{26-54}$ transfection, a fivefold-longer exposure is shown in lanes 15 to 18 of panel B than is shown in lanes 1 to 14. For further details, see the legend to Fig. 2.

17 and 18). Although we do not have enough evidence at this time to determine whether the low activation observed with HA-$4A_{18-54}$ fusion protein is due to impairment of the activation function per se or to secondary effects like protein stability, these results suggest that the minimal sequence encompassing residues 18 to 29 of NS4A (corresponding to amino acids 1675 to 1686 of the polyprotein) is sufficient for proteinase activation. It should be noted that similar results were obtained for NS4A proteins without the HA epitope (data not shown).

Analysis of the hydrophobicity profile of NS4A reveals that this sequence largely corresponds to the second hydrophobic NS4A domain (Fig. 5A). While hydrophobic sequences at the amino terminus might be important in increasing proteinase activation function, the hydrophilic sequence at the carboxy terminus up to amino acid 36 (1693 of the polyprotein) can be deleted without detectable effect on interaction with NS3 and NS3 activation. Comparison of the amino acid sequence of the minimal NS4A domain of our isolate (HCV-G01) with the corresponding regions of several other HCV isolates identified invariant amino acid residues which might play a role in interaction with the proteinase or activation of the proteinase (Fig. 5B): a leucine residue at the beginning of the domain, a threonine-glycine pair followed by three moderately conserved amino acids, an isoleucine, a glycine, and finally an arginine residue close to the end of the domain. To analyze their possible role in NS4A functions, single amino acid substitutions were introduced into $NS4A_{2-54}$ and mutants were coexpressed with the NS4B-5B substrate and the $NS3_{211}$ proteinase domain. To substantiate the results of the NS4A domain mapping, the highly conserved proline residue at position 1696 (i.e., within the dispensable hydrophilic carboxy terminus) was substituted by a leucine residue (1696-P→L) and tested in parallel as described above. As a control, cells expressing only substrate and proteinase were analyzed in parallel (Fig. 6, lanes 1 and 2). Efficient coprecipitation and proteinase activation were observed with the parental NS4A (Fig. 6, lanes 3 and 4), while all substitutions affected these properties to various extents. Slight reduction of coprecipitation and no effect on proteinase activation were found for the substitution outside the essential NS4A domain (1696-P→L). The same phenotype was found for the drastic amino acid exchange close to the center of the minimal domain (1682-I→D). Substitutions at positions 1678 and 1685 by leucine and glutamic acid (1678-G→L and 1685-R→E, respectively) reduced coprecipitation only moderately and had no apparent effect on proteinase activation, suggesting an overall requirement for hydrophobic or charged amino ac-

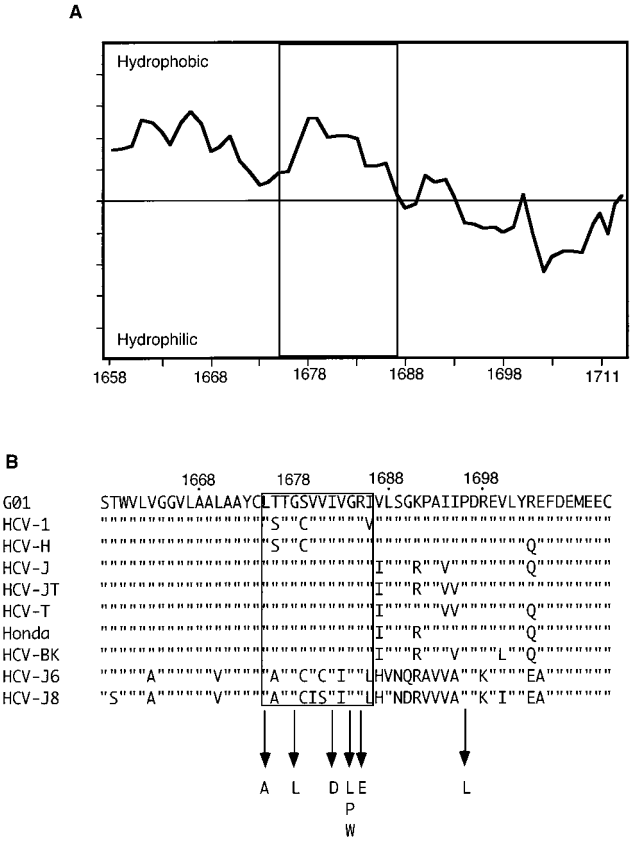

FIG. 5. (A) NS4A hydrophobicity profile as determined by the algorithm of Kyte and Doolittle (25) with a window of 6 amino acids. The minimal NS4A region required to activate the NS3 proteinase for cleavage at the NS4B/5A site is indicated by the box. (B) Alignment of NS4A sequences. The predicted NS4A sequence of our isolate (G01) was aligned with the sequences of HCV isolates given on the left side (for the nomenclature of the individual isolates, see the references in reference 36). Honda is an isolate obtained from the GenBank data base (accession number X61596). Dittos indicate amino acids identical to those in NS4A of our isolate. The minimal proteinase activation region is highlighted by the box. Amino acids selected for mutation analysis are indicated by arrows pointing to the substituting residue.

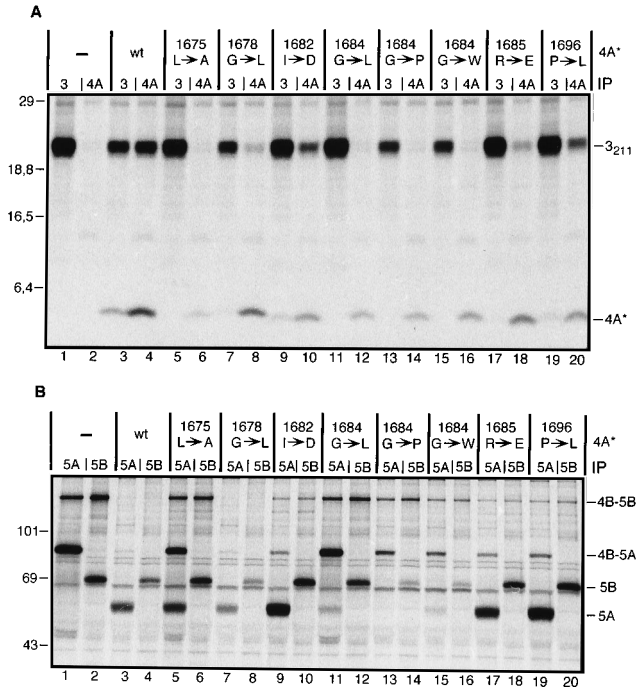

FIG. 6. Mutation analysis of amino acid residues within NS4A conserved between various HCV genotypes. Cells infected with vTF7-3 were transfected with pTM1712-3011 (NS4B-5B), pTM1027-1238 ($NS3_{211}$), and a construct directing the expression of a mutated or parental (wild-type [wt]) NS4A. HCV-specific proteins isolated by immunoprecipitation (IP) with the NS3- or NS4A-specific antiserum were separated by Tricine-SDS-PAGE (A). Proteins recovered with the NS5A- or NS5B-specific antiserum were separated by SDS-PAGE (B). Results of immunoprecipitations from cells expressing substrate and proteinase are shown in lanes 1 and 2 of each panel. For further details, see the legend to Fig. 4.

ids at these positions. No coprecipitation and reduced proteinase activation were found for the leucine substitution at position 1675 (1675-L→A). The most drastic effect was found for the substitutions of the glycine residue at position 1684. For none of these mutants was coprecipitation detected. While for the substitutions by leucine or tryptophan, small amounts of NS5A were found, no NS5A was detected for the substitution by proline, suggesting that steric constraints are the primary reason for loss of NS4A functions. In summary, the overall correlation between impairment of complex formation and reduction of cleavage at the NS4B/5A site shows that interaction between NS3 and NS4A is important for NS3 proteinase activation.

To analyze the influence of NS4A mutations on complex formation and NS3-mediated cleavage at the other sites, the substitution of glycine by proline at position 1684, which had the most marked effect, was introduced into an NS3′-5B polyprotein. This protein was expressed either alone or together with NS4A, and HCV-specific proteins were isolated under nondenaturing conditions as described above. As a control, cells expressing an NS3′-5B polyprotein with an unaltered NS4A were analyzed in parallel. As shown in Fig. 7, mature NS5B and small amounts of NS3 but no NS4A, NS4B, or NS5A were found in cells expressing the NS3′-5B polyprotein with the defective NS4A (lanes 5 to 8). Instead, a series of processing intermediates corresponding to NS3′-5A, NS4AB-5A, NS4B-5A, and NS3′-4A were observed, indicating inefficient cleavage at the NS4A-dependent sites. Very small amounts of NS3 were coprecipitated with the NS4-specific antiserum consistent with the defect described above for this NS4A mutant (lane 6). When NS4A was coexpressed, all mature cleavage products as well as increased amounts of NS3 were detected (lanes 9 to 12) and NS3 was coprecipitated with NS4A (lane 10). Thus, destabilization of the complex by an NS4A mutation impaired proteolytic cleavage at all NS4A-dependent sites and could be restored by NS4A provided in *trans.*

Amino-terminal NS3 sequences are important for complex formation and NS4A-mediated activation. We and others have recently shown that amino-terminal NS3 deletions greatly reduce cleavage at the NS4B/5A site without significantly affecting processing between NS5A and NS5B (5, 13). Since NS4A is absolutely required for efficient cleavage between NS4B and NS5A but not for processing between NS5A and NS5B, amino-terminal NS3 sequences presented prime candidates for interaction with NS4A. To test this hypothesis, cells were transfected with constructs directing the expression of the NS4B-5B substrate, $NS4A_{2-54}$, and the proteinase domain either with an unaltered or truncated amino terminus. For optimal separation in the low- and high-molecular-weight range, half of each sample was analyzed by Tricine-SDS-PAGE and SDS-PAGE in parallel (Fig. 8A and B, respectively). As a control, cells expressing only the substrate (Fig. 8, lanes 1 to 4), substrate plus $NS4A_{2-54}$ (lanes 5 to 8), or substrate plus the parental proteinase (lanes 9 to 12) were examined in parallel. As shown in Fig. 8B (lanes 11 and 12) in the absence of NS4A, cleavage of the substrate occurred only at the NS5A/5B site. NS5A and the NS3-NS4A complex were clearly detected in cells expressing in addition NS4A (Fig. 8B, lane 15, and Fig. 8A, lanes 13 and 14, respectively). Removal of seven amino acids from the amino terminus of NS3 significantly destabilized the complex, as seen by the reduced amount of NS3 coprecipitated with the NS4A-specific antiserum and the small amounts of NS4A coprecipitated with the NS3-specific antiserum (Fig. 8A, lanes 17

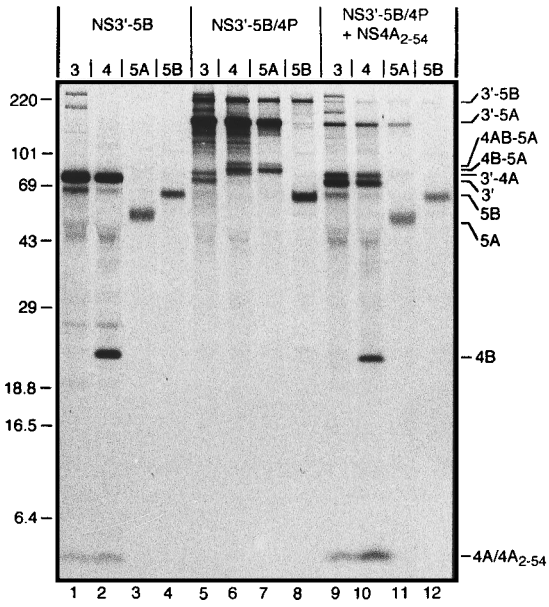

FIG. 7. The proline substitution in NS4A affects cleavage at the NS4A-dependent sites. Infected cells were transfected with pTM1007-3011/4P alone (lanes 5 to 8) or together with $NS4A_{2-54}$ (lanes 9 to 12). Proteins were isolated under nondenaturing conditions by immunoprecipitation (IP) with the antiserum given above each lane and analyzed by Tricine-SDS-PAGE. As a control, proteins isolated from cells transfected with pTM1007-3011 were analyzed in parallel (NS3′-5B; lanes 1 to 4). For further details, see the legend to Fig. 2.

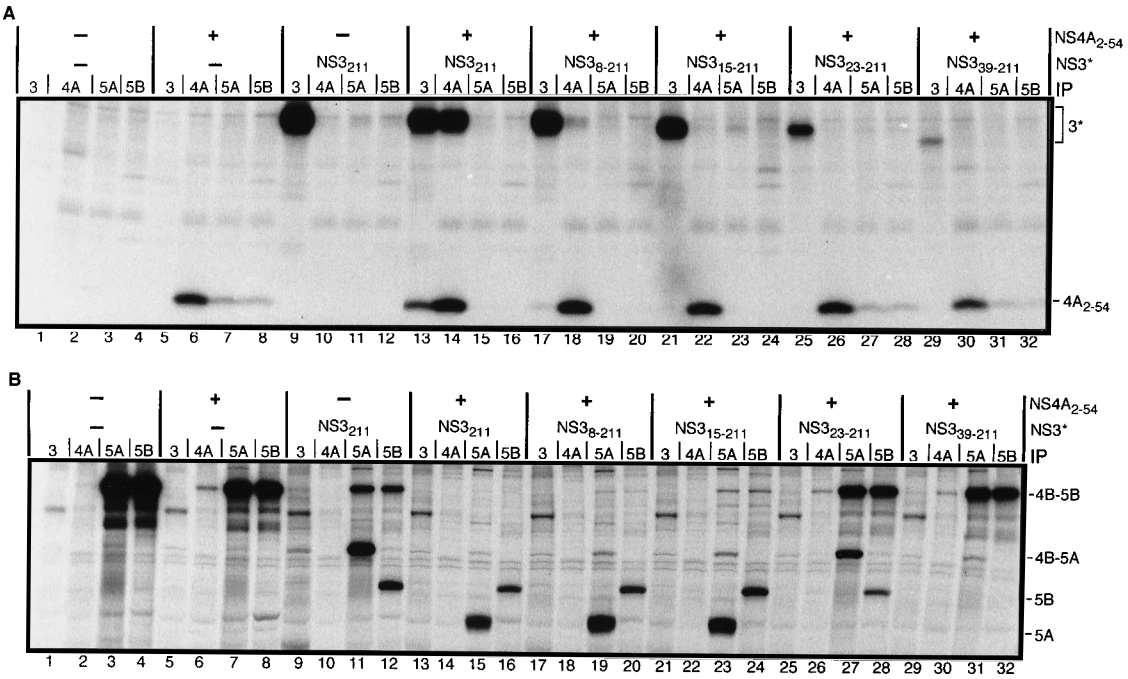

FIG. 8. Amino-terminal sequences of NS3 are important for interaction with NS4A. BHK-21 cells previously infected with vTF7-3 were transfected with pTM1712-3011 (NS4B-5B) either alone (lanes 1 to 4) or with pTM1659-1711 ($NS4A_{2-54}$; lanes 5 to 8), pTM1027-1238 ($NS3_{211}$; lanes 9 to 12), or pTM1712-3011 plus pTM1659-1711 plus one of the indicated NS3 constructs (NS3*; lanes 13 to 32). Following metabolic labeling, HCV-specific proteins were isolated by immunoprecipitation (IP) under nondenaturing conditions with the indicated antiserum. One half of the samples were separated by electrophoresis on Tricine–11% polyacrylamide gels (A), and the other half were separated on SDS–11% polyacrylamide gels (B). Identification of HCV proteins is given on the right of each gel. Only the parts of the gels with sufficient resolution of HCV-specific proteins are shown. For further details, see the legend to Fig. 2.

and 18). Cleavage at the NS4B/5A junction apparently was not affected (Fig. 8B, lanes 19 and 20). Slightly increased amounts of uncleaved precursor and NS4B-5A intermediate, indicating a reduced processing efficiency, were found with the proteinase lacking 15 amino acids (Fig. 8B, lanes 23 and 24). Interaction of this truncated NS3 with NS4A was reduced below the detection limit (Fig. 8A, lanes 21 and 22). Proteinase activation by NS4A was no longer detectable when 22 amino acids were deleted and only uncleaved NS4B-5A and mature NS5B were found along with clearly increased amounts of uncleaved precursor (Fig. 8B, lanes 27 and 28). However, given the small amount of proteinase detected currently, we cannot ascertain whether the overall reduction of proteolytic activity is due exclusively to a reduction of the activity per se or at least to some extent to the lower expression or stability of this protein (Fig. 8A, lane 25). Similar results were found for the NS3 proteinase lacking 38 amino-terminal amino acids (Fig. 8, lanes 29 to 32). Beside interaction with NS3, NS4A was also coprecipitated with the NS4B-5B substrate by using NS5A- or NS5B-specific antiserum (Fig. 8A, lanes 7 and 8) and the substrate was coprecipitated with NS4A by using the NS4A-specific antiserum (Fig. 8B, lane 6). This coprecipitation was no longer detectable in cells expressing NS3 proteins which efficiently cleaved the substrate (e.g., Fig. 8A, lanes 19 and 20) but was found in cells expressing truncated NS3 proteins with low substrate cleavage, suggesting that NS4A can also interact with the NS4B-5B substrate (Fig. 8A, lanes 27, 28, 31, and 32). This interaction appears to be much weaker than the one observed with NS3 because in cells expressing the NS4B-5B substrate, NS4A, and an enzymatically inactive $NS3_{211}$ proteinase, NS4A was coprecipitated with NS3 but not with the substrate (data not shown).

The results described so far for the NS3 deletion mutants imply that proteins with amino-terminal NS3 truncations significantly impaired for interaction with NS4A can still be activated ($NS3_{8-211}$ and $NS3_{15-211}$). However, since the buffer we used for cell lysis and immunoprecipitation contained 0.1% SDS, it was possible that these truncated proteinases could still interact with NS4A intracellularly but that these complexes were sensitive to our lysis conditions. Since our antisera are directed against denatured antigens, HCV-specific proteins were not or only poorly precipitated from cell lysates prepared exclusively with nonionic detergents. Therefore, we used a modified lysis buffer containing reduced amounts of SDS for immunoprecipitation of HCV proteins from cells expressing $NS4A_{2-54}$ and either the parental $NS3_{211}$ or one of the NS3 proteins with amino-terminal truncations. Under these conditions the levels of coprecipitation of NS4A between the complete $NS3_{211}$ proteinase and the truncated $NS3_{8-211}$ proteinase were comparable (Fig. 9, lanes 1 to 4). Coprecipitation was clearly reduced for the $NS3_{15-211}$ proteinase but still detectable as seen by the small amounts of NS4A in the immunoprecipitation with the NS3-specific antiserum (lane 5), showing that this truncated proteinase still retained the ability to interact with NS4A. No coprecipitation was found for the $NS3_{23-211}$ proteinase, consistent with the inability to activate this enzyme for cleavage at the NS4B/5A site.

DISCUSSION

Previous studies have shown that NS4A is a proteinase cofactor required for cleavage at the NS3/4A, NS4A/4B, and NS4B/5A sites and enhancing processing efficiency between NS5A and NS5B (5, 12, 27). In this work we analyzed the mechanism of proteinase modulation by NS4A and found that NS4A most likely exerts its effect by formation of a stable

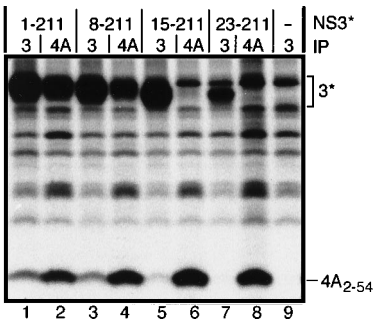

FIG. 9. Interaction between proteinases with amino-terminal NS3 truncations and NS4A. vTF7-3-infected BHK-21 cells were transfected with vector pTM1-2 (lane 9) or pTM1659-1711($NS4A_{2-54}$) and one of the given proteins with NS3 truncations and HCV-specific proteins were isolated by immunoprecipitation under nondenaturing conditions with a buffer containing small amounts of sodium deoxycholate and SDS (see Materials and Methods). Samples were analyzed by Tricine-SDS-PAGE (11% polyacrylamide).

complex with the NS3 proteinase. Several observations support this conclusion. (i) Deletions into the NS4A minimal activation domain abolished NS3-NS4A interaction and impaired cleavage at the NS4B/5A site. (ii) For several NS4A mutations within this domain, a correlation between loss of interaction and loss of processing at this site was found. (iii) No or reduced cleavage at the NS4A-dependent sites but no significant effect on *trans* cleavage between NS5A and NS5B was found for an NS4A mutant defective for complex formation. (iv) Deletion of amino-terminal NS3 sequences affecting complex formation impaired processing between NS4B and NS5A. Thus, interaction between NS3 and NS4A appears to be important for full proteolytic activity at the NS4A-dependent sites.

The results described here are reminiscent of the situation of flaviviruses. For dengue virus 2 and yellow fever virus, it is generally assumed that the active proteinase is a heterodimer composed of NS3 and NS2B, which is located amino terminally of NS3 and released from the polyprotein precursor by intramolecular cleavage (2, 6, 14). Mutations in the NS2B domain destabilizing the complex also impair proteinase activity (6). However, apart from this close similarity, it should be noted that flavivirus NS2B is absolutely essential for NS3 activity, while for HCV the NS3 proteinase retains enzymatic activity in the absence of NS4A. For pestiviruses, the results of Wiskerchen and Collett (41) suggest that p10, which with respect to size and localization within the polyprotein is probably the functional analog of HCV NS4A, might be a proteinase cofactor. By using a substrate similar to NS5A-5B, cleavage was detected only for a proteinase containing carboxy-terminal p10 and part of p32 (corresponding to NS4B). However, when p10 was expressed as part of the substrate, cleavage at this site was observed even for a proteinase lacking downstream sequences, suggesting that p10 can be provided in *cis* (together with the proteinase) or in *trans* (together with the substrate) to restore cleavage at this site. Similar results have been described for HCV (5, 12, 27).

The mechanism by which NS4A modulates proteinase activity is not known. Interaction between NS3 and NS4A may be required for localization of NS3 to the endoplasmic reticulum membrane where most of the NS proteins are found (21, 34, 38). In this case NS4A would increase the local proteinase concentration, thereby enhancing substrate cleavage. Furthermore, the half-life of NS3 is greatly influenced by NS4A because the rapid degradation of NS3 expressed as a single entity in cell culture can be prevented by coexpression of NS4A (38). Thus, membrane attachment of NS3 via NS4A might have a dual function: to increase the proteinase concentration close to the substrate and to sequester the proteinase from rapid cellular degradation. Given the fact that cleavage at the NS5A/5B site occurs in the absence of NS4A and that the proteinase can be activated in vitro independently of the presence of membranes (28), membrane association of the proteinase and elongation of its half-life are probably not the main mechanisms of NS3 activation. Instead, NS4A may have a function similar to the one found for propeptides of several bacterial serine proteinases like α-lytic proteinase or subtilisin (3, 43). These propeptides are essential for proteinase activation, they are cleaved off the proteinase domain by an intramolecular reaction, they can activate the proteinase in *cis* or in *trans*, and the prosequence can exert its activation function even when it remains fused to the enzyme. The similarities to NS4A are obvious. NS4A is required for full NS3 activity (5, 12, 27), it is removed from the proteinase by an intramolecular cleavage at the NS3/4A site (5, 27, 39), it can activate in *cis* or in *trans* (5, 12, 27), and proteinase activation does not require prior liberation of NS4A from the polyprotein (12). Propeptides of the bacterial enzymes mentioned above assist the folding of their cognate proteinase, and when folding has been completed, they become dispensable (3, 43). Given the similarities, it seems plausible that NS4A plays an important part in folding of the NS3 proteinase. However, in contrast to the propeptides, NS4A is not absolutely required for NS3 activity, which suggests that in this model, proteinase structure acquired in the absence of the cofactor is sufficient for cleavage at the NS5A/5B site (but not optimal, at least for some substrates) and that interaction with NS4A would alter the conformation of the enzyme in a manner essential for efficient processing at the NS4A-dependent sites. Our finding that the NS3 proteinase lacking the 14-amino-terminal residues can be activated to a level comparable with that of the parental NS3 but exhibits significantly impaired interaction with NS4A suggests that a weak overall association between NS3 and NS4A is sufficient for activation. In this case the stability of the complex observed under normal conditions might not be required for proteinase activation but perhaps for membrane localization of NS3. Since the carboxy-terminal NS3 domain most likely constitutes a helicase (37), membrane attachment of NS3 could be important in incorporation of the helicase into a stable membrane-associated replication complex.

Beside modulating the folding of the proteinase, the coprecipitation of NS4A with the NS4B-5B substrate suggests that NS4A can also interact with the substrate. Such an interaction might be important in facilitating proteinase-substrate interaction, for instance, to unfold locally the region around the scissile bonds to render the cleavage site accessible to the enzyme or to increase the stability of the proteinase-substrate interaction, particularly at the NS4A-dependent sites. However, whether interaction of NS4A with the substrate plays an important role in cleavage remains to be determined.

The nature of the interaction between NS3 and NS4A is not known at this time. Certainly as shown in this study and in two recent reports (13, 33), amino-terminal NS3 sequences are required. Since deletion of these sequences did not significantly affect cleavage at the NS5A/5B site, the overall structure of these variant NS3 proteins was not grossly altered. Rather, amino-terminal NS3 sequences appear to constitute an autonomous NS4A binding domain (33).

We have recently shown that deletion of amino-terminal NS3 sequences abolishes cleavage at the NS4B/5A site without significant effect on processing at the other *trans* cleavage sites (5). Although the results described in this report confirm this

observation by using stable recombinant vaccinia viruses expressing various NS3 proteins, in the first study (5) we found a strong reduction of processing at the NS4B/5A site when only 7 amino acids were deleted, while in this report by using the vaccinia virus-T7 hybrid system, 7 and even 14 residues could be removed without detectable effects. Because the proteinases expressed in both systems have the same amino acid sequence, the most likely reason for this discrepancy is the assay system. Using the T7 system, Faila and coworkers found that cleavage at the NS4B/5A site by a proteinase lacking 15 amino-terminal residues (amino acids 1042 to 1237 of the polyprotein) was severely reduced and that cleavage at this site by a proteinase lacking 28 residues (amino acids 1055 to 1237) was detectable only when large amounts of plasmid were transfected (13). In contrast, efficient cleavage at this site was observed for a proteinase lacking 22 amino-terminal residues (amino acids 1049 to 1215) when coexpressed under control of the human cytomegalovirus immediate-early promoter with NS4A and an artificial substrate composed of 30 amino acids spanning the NS4B/5A cleavage site fused to the amino terminus of the *E. coli* dihydrofolate reductase (38). On the other hand, the same proteinase domain fused to dihydrofolate reductase and coexpressed with NS4A inefficiently cleaved an NS4B-5A substrate (33). The reason for these differences is not clear but is most likely due to differences in the expression system, assay conditions, or primary sequences of the proteinases or substrates. This issue can be clarified only with the development of an appropriate in vitro cleavage system.

A minimal sequence of 12 amino acids mapping close to the center of NS4A (amino acids 18 to 29) was found to be essential for proteinase activation and, as indicated by mutation analysis, also for complex formation. Although highly conserved in various HCV genotypes, the carboxy-terminal 19 residues are not required for complex formation and proteinase activation, suggesting that this sequence has another function. Proper folding of the NS4A/4B processing site is a likely possibility. This result is in agreement with two recent reports mapping the NS4A region important for cofactor activity to amino acids 21 to 30 (38) or 21 to 33 (28). Furthermore, it was shown that a peptide corresponding to amino acids 22 to 34 of NS4A can activate the proteinase in vitro (24, 28). Although other possibilities exist, these results suggest a stable interaction between the central NS4A domain and the amino-terminal domain of NS3.

Modulation of proteinase activity by cofactors is also found for other viral systems. For adenovirus, an 11-amino-acid peptide derived from the structural protein pVI forms a disulfide-linked heterodimer with the proteinase and this interaction is essential for enzyme activation (40). For poliovirus, the 3C proteinase is modulated by the 3D RNA-dependent RNA polymerase (23, 42). 3C is sufficient for cleavage within P2 and P3, but processing within the capsid protein precursor P1 is catalyzed by a 3CD proteinase. An auxiliary function of 3D discussed is to facilitate enzyme-substrate interaction by direct interaction of 3D with the P1 precursor (42). For Sindbis virus, a member of the alphaviruses, cleavage site preference of the nsP2 proteinase is modulated by the flanking nonstructural proteins nsP1 and nsP3, resulting in a temporal regulation of polyprotein processing (8).

The finding that interaction of the NS3 proteinase with NS4A is important for full proteolytic activity encourages the development of a new therapeutic concept. As shown recently for yellow fever virus, mutations in NS2B blocking interaction with the NS3 proteinase and proteinase activation are deleterious for virus infectivity (6). By analogy, it is likely that HCV NS4A, because of its important role for polyprotein maturation, is also essential for virus replication. Thus, beside the development of inhibitors targeted to the active site of the proteinase, inhibition of the NS3-NS4A interaction may provide an alternative for the development of therapeutic agents and methods urgently needed to control this insidious disease.

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