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An Amino-Terminal Domain of the Hepatitis C Virus NS3 Protease Is Essential for Interaction with NS4A

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Hepatitis C virus (HCV) genomic RNA is translated into a large polyprotein that is processed into structural and nonstructural proteins. Processing at the N termini of several nonstructural proteins requires sequences contained in both NS3 and NS4A. NS3 contains a serine protease, whereas the function of NS4A in proteolysis is yet to be determined. By using the vaccinia virus-T7 hybrid expression system to transiently express HCV polypeptides in HeLa cells, we studied the effect of several N-terminal and C-terminal deletions of HCV NS3 on the processing activity at all the downstream cleavage sites. In this way, we have delineated the minimal domain of NS3 required for the serine protease activity associated with this protein. In addition, we demonstrate the formation of a stable complex between NS3 and NS4A: analysis of the deletion mutants reveals a region at the N terminus of NS3 that is necessary for both complex formation and modulation of the proteolytic activity by NS4A but not for the NS4A-independent serine protease activity of NS3.

Hepatitis C virus (HCV) is considered to be the major etiologic agent of both parenterally transmitted and sporadic non-A, non-B hepatitis (9, 27). In all, hepatitis C infection is estimated to affect around 50 million people worldwide. About a half of the afflicted individuals become chronically infected, and liver cirrhosis develops in approximately 20% of this group (29). In addition, increased incidence of hepatocellular carcinoma in patients with non-A, non-B hepatitis suggests that HCV may play a role also in the process of hepatocarcinogenesis (8, 34).

The molecular cloning of the HCV genome (9) has opened up new avenues of study. It is now known that HCV is an enveloped virus which contains a positive-stranded RNA genome of about 9,500 nucleotides (10, 26, 36). HCV has a genome and polyprotein organization similar to that of human flaviviruses and animal pestiviruses. On the basis of their genomic structure and virion properties, HCV, pestiviruses, and the flaviviruses have been classified as three genera in the *Flaviviridae* family (16). The viral genome of HCV contains a single open reading frame, which encodes a polyprotein of about 3,000 amino acids (30). This polyprotein undergoes proteolytic processing in the cytoplasm or in the endoplasmic reticulum of the infected cell to give rise to at least nine proteins (20, 21, 37). Those liberated from the amino terminus of the polyprotein by the action of host proteases are thought to be the structural proteins. These include the nucleocapsid C protein (21 kDa) and two putative envelope glycoproteins, E1 (37 kDa) and E2/NS1 (61 kDa). The signal peptidase of the endoplasmic reticulum is responsible for generating the N termini of E1, E2, and possibly NS2 (21), whereas it has been recently established that a novel virus-encoded metalloprotease is responsible for the cleavage between NS2 and NS3 (18, 22). The C-terminal remainder of the HCV polyprotein is further processed to give rise to NS3 (68 kDa), NS4A (6 kDa), NS4B (26 kDa), NS5A (56 kDa), and NS5B (65 kDa) proteins. It has been demonstrated that the proteolytic cleavage at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions is mediated by a virus-encoded serine protease, contained within NS3, that bears homology to the small cellular proteases of the trypsin superfamily (3, 12, 19, 37). It has also been established that the NS3 serine protease is necessary but not sufficient for efficient processing at all the downstream cleavage sites. In addition to NS3, the NS4A protein is required for cleavage at the NS3/4A and NS4B/5A sites, and it increases the efficiency of cleavage at the NS5A/5B and NS4A/4B junctions (4, 14).

The need for a protease cofactor is reminiscent of what is observed in flaviviruses: in dengue virus and yellow fever virus, NS2B is required to facilitate efficient proteolytic processing of the nonstructural region of the viral polyprotein by NS3 (1, 6, 15, 28, 32). In contrast to HCV NS4A, flavivirus NS2B is located at the N terminus of NS3. In spite of their different topological arrangements, HCV NS4A and flavivirus NS2B share several features: both proteins are required as effectors of protease activity, can function in *cis* or in *trans*, and are liberated from their respective catalytic domains by an intramolecular cleavage. A stable physical interaction between flavivirus NS3 and NS2B has been demonstrated, and mutations in NS2B that affect complex formation also impair proteolytic processing by NS3 (1, 15), suggesting that the two proteins may associate and that the active form of the protease is a heterodimer. Similar detailed studies on the interaction between HCV NS3 and NS4A are not yet available.

Exploiting the vaccinia virus-T7 hybrid expression system to transiently express HCV polypeptides in HeLa cells, we analyzed the effects of several N-terminal and C-terminal deletions of HCV NS3 on the processing activity at all the downstream cleavage sites. Our results implicate a 169-residue fragment of NS3 as the minimal domain responsible for full proteolytic activity. In addition, we observe complex formation in vivo between NS3 and NS4A: analysis of the deletion mutants reveals a region at the extreme N terminus of NS3 that is necessary both for efficient complex formation and for modulation of the proteolytic activity by NS4A but not for the NS4A-independent serine protease activity of NS3.

MATERIALS AND METHODS

Cells and virus. HeLa cells, originally obtained from the American Type Culture Collection, were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Vaccinia virus VTF7-3 (17) was grown in RK13 cells cultured in minimum essential medium containing 10% fetal calf serum.

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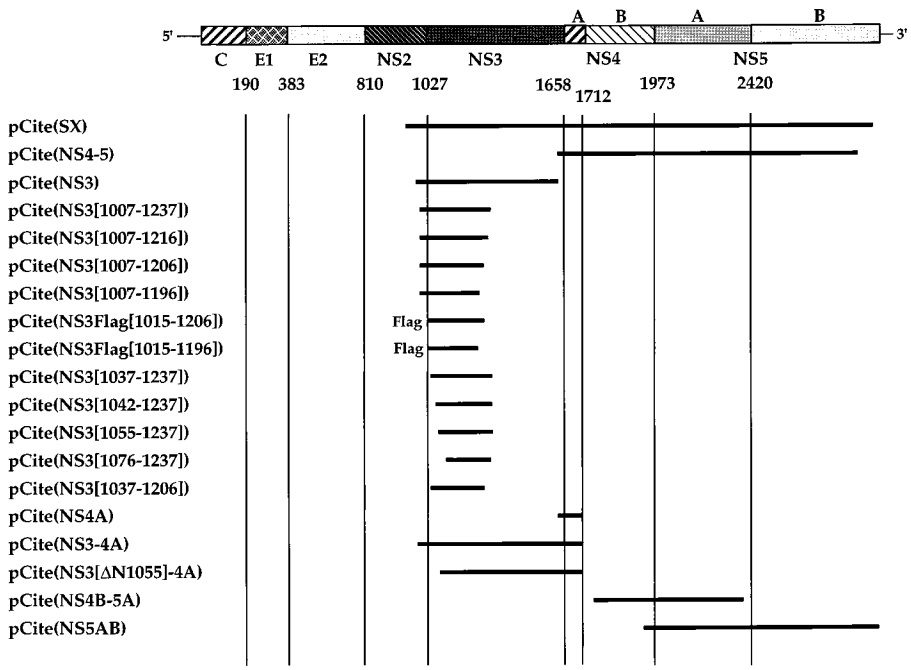

FIG. 1. Schematic representation of the recombinant expression plasmids used in this study. The organization of the viral polyprotein is shown at the top. The vertical bars indicate the boundaries between the different proteins. The number at the top of each bar indicates the position of the N-terminal amino acid of the following protein within the viral polyprotein. The HCV polyprotein portions expressed by the different constructs are shown below; the annotation of the corresponding plasmids is shown on the left. "Flag" indicates an octapeptide marker with the amino acid sequence DYKDDDDK (23).

Construction of recombinant plasmids. Appropriate DNA fragments derived from HCV-BK (36) cDNA were inserted downstream of the 5′ untranslated region of encephalomyocarditis virus and under a T7 promoter in the pCite-1 vector (Novagen) (13, 25), in the appropriate translational reading frame and followed by a termination codon. This expression vector contains the encephalomyocarditis virus internal ribosome entry site (25), which promotes efficient and cap-independent translation of mRNA transcribed from the T7 promoter (13). All the plasmids described below were obtained by using standard recombinant DNA technology (33).

pCite(SX) and pCite(NS4-5) contain HCV cDNA fragments spanning nucleotides 3303 to 9416 (amino acid residues 991 to 3010) and nucleotides 5281 to 9071 (amino acid residues 1651 to 2921), respectively. pCite(NS3) contains a cDNA fragment comprised between nucleotides 3351 and 5171 (amino acid residues 1007 to 1615). Construction of these plasmids was reported in reference 37. pCite(NS4A) bears a cDNA fragment spanning nucleotides 5281 to 5465 of the HCV genome (amino acid residues 1649 to 1711). pCite(NS5AB) contains a cDNA fragment comprised between nucleotides 6224 and 9400 (amino acid residues 1965 to 3010). pCite(NS4B/5A) contains a cDNA fragment comprised between nucleotides 5652 and 7467 (amino acid residues 1774 to 2380). Construction of these plasmids was reported in reference 14.

The C-terminal deletion of pCite(NS3) termed pCite[NS3(1007-1237)] contains the HCV sequence from nucleotides 3351 to 4043 and was generated by partial digestion of pCite(NS3) with *Sca*I. The plasmid was then circularized with the insertion of a double-stranded oligonucleotide containing a stop codon in all reading frames (TAA CTA GTA GGA TCC TAC TCA GTT A). The other three C-terminal deletions, pCite[NS3(1007-1216)], pCite[NS3(1007-1206)], and pCite[NS3(1007-1196)], contain nucleotides 3351 to 3979, 3351 to 3950, and 3351 to 3920, respectively, and were generated by amplification of the appropriate cDNA fragments by PCR and subsequent cloning in the pCite vector.

pCite(NS3Flag[1015-1206]) and pCite(NS3Flag[1015-1196]) were generated by digestion of pCite[NS3(1007-1206)] and pCite[NS3(1007-1196)], respectively, with *Xma*III. The plasmid was then circularized with the insertion of a double-stranded oligonucleotide containing the sequence ACC ATG GAC TAC AAG GAC GAC GAT GAC AAA GGA TC.

pCite[NS3(1037-1237)] (nucleotides 3441 to 4041), pCite[NS3(1042-1237)] (nucleotides 3457 to 4041), pCite[NS3(1055-1237)] (nucleotides 3494 to 4041), and pCite[NS3(1076-1237)] (nucleotides 3557 to 4041) were constructed by BAL 31 nuclease treatment on pCite[NS3(1007-1237)]. Briefly, pCite[NS3(1007-1237)] was linearized with *Apa*I and treated with BAL 31 nuclease (33). A set of DNA fragments was then excised by using the enzyme *Pst*I and recloned in the *Nco*I-*Pst*I sites of pCite[NS3(1007-1237)], after filling in the *Nco*I extremity with the Klenow polymerase. Several clones were sequenced, and those of interest were selected.

pCite[NS3(1038-1206)] contains the HCV sequence from nucleotides 3444 to 3950 and was generated by cloning the *Bst*XI-*Bst*XI 1,376-bp fragment of pCite[NS3(1038-1238)] into the *Bst*XI sites of pCite[NS3(1007-1206)]. pCite[NS3(1038-1238)] contains nucleotides 3444 to 4041 and was obtained by BAL 31 nuclease digestion as described above.

pCite(NS3-4A) contains a cDNA fragment comprising the region between nucleotides 3303 and 5465 of HCV cDNA (14). The resulting construct codes for the portion of the HCV polyprotein comprised between amino acids 991 and 1711. To construct pCite(NS3[ΔN1055]-4A) (nucleotides 3494 to 5465, amino acids 1055 to 1711), an *Eco*RI-*Xma*III fragment was excised from pCite[NS3(1055-1237)] and religated in the same sites of pCite(NS3-4A).

All HCV-derived expression plasmids relevant for this study are graphically shown in Fig. 1.

Transient expression of HCV proteins and preparation of labelled extracts. HeLa cells seeded at a density of 4×10^5 cells per 6-cm-diameter plate were infected with vaccinia virus vTF7-3, a recombinant vaccinia virus that supports the synthesis of bacteriophage T7 polymerase in the cytoplasm of infected cells (17), at a multiplicity of 5 PFU per cell and transfected by the calcium phosphate method as previously described (37). Cell proteins were pulse-labelled with [^{35}S]methionine for various times. Cell lysates from transient expression experiments were prepared under denaturing conditions as described previously (31). Immunoprecipitations were conducted on denatured extracts with the HCV region-specific rabbit polyclonal antisera described previously (31, 37) or with immobilized M2 anti-Flag antibody purchased from International Biotechnologies Inc.

Lysates for analysis of protease complex formation were prepared under nondenaturing conditions in buffer N {40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH (pH 8), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% 3-[(3-cholamidinopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 20% glycerol}. Following solubilization of cell membranes in buffer N, the lysate was centrifuged at 10,000 rpm for 10 min in an Eppendorf Microfuge, and the pellet was discarded. Immunoprecipitations were then conducted on nondenatured extracts by using HCV region-specific rabbit polyclonal antisera (31, 37) as follows. Antiserum (10 μl) was incubated in 400 μl of IPB_{150} (20 mM Tris-Cl [pH 8.0], 150 mM NaCl, 1% Triton) with 20 μl of protein A (PA)-Sepharose for 1 h at 4°C. The PA-Sepharose beads were pelleted by centrifugation, washed twice with 400 μl of buffer N, resuspended in 400 μl of buffer N, and incubated for an additional hour at 4°C with 20 μl of nondenatured cell lysate. All reactions were performed with constant mixing on a end-over-end rotator. The PA-Sepharose suspension was then layered on 0.9 ml of 0.5× buffer N containing 30% sucrose and pelleted by centrifugation at 2,000 rpm in an Eppendorf Microfuge for 10 min at room temperature. The pellet was resuspended in 400 μl of buffer N and then washed twice with the same buffer and

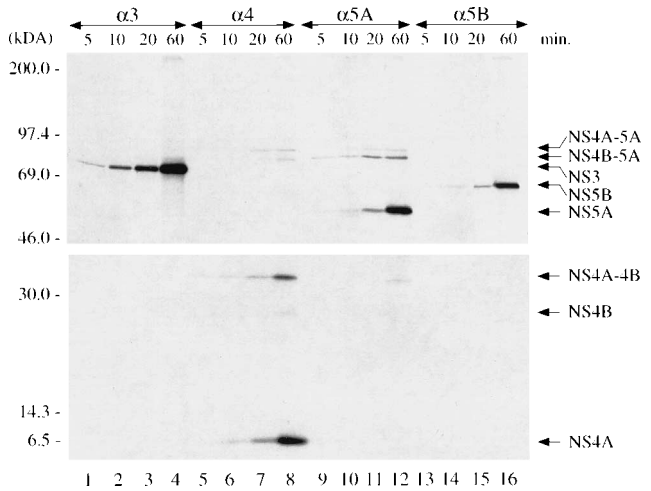

FIG. 2. Kinetic analysis of the NS3-NS5B polyprotein processing by pulse-labelling. Cells were transfected with plasmid pCite(SX) and 4 h later labelled with [^{35}S]methionine for the times indicated. Cell lysates were prepared and immunoprecipitated with HCV-specific rabbit antisera. Lanes 1 to 4, anti-NS3; lanes 5 to 8, anti-NS4; lanes 9 to 12, anti-NS5A; lanes 13 to 16, anti-NS5B. Immunoprecipitated proteins were separated by electrophoresis on an SDS–12% (upper panel) or 14% (lower panel) polyacrylamide gel. HCV-specific proteins are indicated by arrows on the right. The sizes of molecular weight marker proteins are indicated on the left.

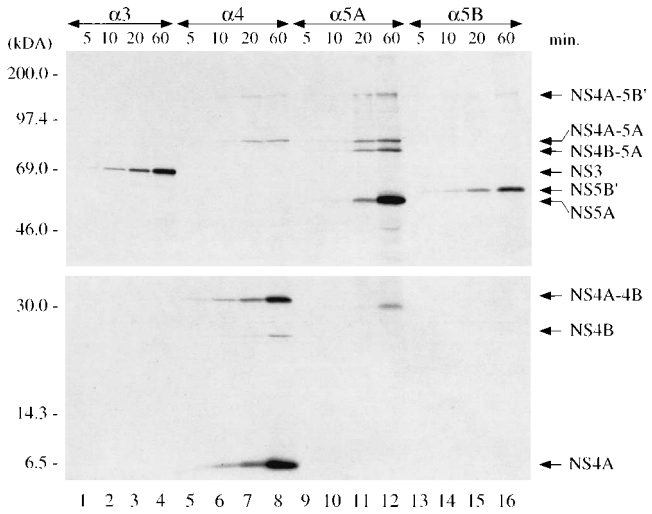

FIG. 3. Kinetic analysis of NS4A-NS5B polyprotein processing in *trans* by the NS3 protease. Cells were cotransfected with the plasmids pCite(NS4-5) and pCite(NS3). Four hours after transfection, cells were labelled with [^{35}S]methionine for the times indicated. Cell lysates were prepared and immunoprecipitated with HCV-specific rabbit antisera. Lanes 1 to 4, anti-NS3; lanes 5 to 8, anti-NS4; lanes 9 to 12, anti-NS5A; lanes 13 to 16, anti-NS5B. Immunoprecipitated proteins were separated by electrophoresis on an SDS–12% (upper panel) or 14% (lower panel) polyacrylamide gel. HCV-specific proteins are indicated by arrows on the right. The sizes of molecular weight marker proteins are indicated on the left.

once with phosphate-buffered saline. The sample was then resuspended in 20 μl of sodium dodecyl sulfate (SDS) sample buffer, heated at 95°C for 5 min, and centrifuged for 5 min. The supernatant was loaded on an SDS-polyacrylamide gel.

RESULTS

Kinetics of processing of nonstructural proteins by NS3 in *cis* and in *trans*. We wished to establish a cellular assay based on the vaccinia virus-T7 expression system that would enable us to assess the proteolytic activity of a variety of N-terminal and C-terminal deletion products of the NS3 protein. Normally, the NS3 protein is part of the HCV polyprotein and therefore linked to its substrates. However, we previously showed that NS3 provided in *trans* can mediate cleavage of a polyprotein precursor containing all of the downstream nonstructural proteins, NS4A to NS5B (37). We decided to evaluate the possibility of using this latter polyprotein fragment as a substrate in a *trans*-cleavage assay, primarily to avoid the complication of engineering NS3 mutants in the context of the whole HCV polyprotein. For this reason, we performed pulse-labelling experiments in which we compared the efficiency of processing by NS3 supplied in *trans* to that observed when NS3 is physically linked to its substrate (in *cis*).

We analyzed, at different labelling times, the protein products of pCite(SX); we have previously shown that the polyprotein encoded by this plasmid properly reflects all of the cleavage events mediated by the NS3 proteins. The results of this experiment are shown in Fig. 2. The mature forms of NS3 (Fig. 2, lanes 1 to 4) and NS5B (Fig. 2, lanes 13 to 16) are detectable after 5 min and continue to accumulate for 60 min without evidence of high-molecular-weight precursors, indicating that the cleavages at the NS3/4A and NS5A/5B junctions are very rapid and efficient events. NS4A (Fig. 2, lanes 5 to 8) and NS5A (Fig. 2, lanes 9 to 12) were also detectable between 5 and 10 min of labelling and accumulated at longer times, but in addition to the mature processed products, higher-molecular-weight proteins that correspond in molecular weight and immunoreactivity to the NS4A-NS4B, NS4A-NS5A, and NS4B-NS5A precursors are visible (Fig. 2). In particular, the NS4A-NS4B precursor (Fig. 2, lanes 5 to 8) appeared to be very abundant. Despite the relatively early appearance of NS4A, NS4B (Fig. 2, lanes 5 to 8) appeared only late and after prolonged exposure. This observation could be explained by either the low reactivity with the anti-NS4 antiserum or the inherent instability of this protein (4) rather than by delayed cleavage at the NS4A/4B site.

Next, we analyzed by pulse-labelling experiments the protein products obtained by cotransfecting in HeLa cells plasmids pCite(NS4-5) and pCite(NS3). It should be noted that the former plasmid contains a truncated form of NS5B (NS5B′). The results of the time course are shown in Fig. 3. Interestingly, the kinetics of production of the mature forms of NS4A, NS4B, NS5A, and NS5B′, as well as those of their precursors, are virtually identical to those observed when NS3 was synthesized as part of the precursor polyprotein (Fig. 2). The anti-NS4 antiserum preparation used in this and in the following experiments differs from that used in the experiment of Fig. 2 in that it did not react with the 88-kDa protein precursor that we previously identified as NS4B-5A.

Analysis of the NS3 deletion mutants and mapping of the minimal protease domain. Previous studies of HCV polyprotein processing have indicated that NS3 contains a serine protease involved in the maturation of HCV nonstructural proteins (3, 19, 37). Analysis of the sequence similarity between HCV NS3 and other well-characterized cellular and viral serine proteases has suggested that the protease domain is contained within the N-terminal third of the protein (5). To map precisely the boundaries of the protease domain, we constructed plasmids encoding a series of N-terminal and C-terminal deletion mutants of NS3 (Fig. 1). The shortest C-terminal deletion mutant analyzed, NS3[1007-1196], was not recognized by our anti-NS3 antiserum preparation. Therefore a Flag epitope sequence (DYKDDDDK [23]) was fused at the N terminus of this mutant. This allowed us to immunoprecipi-

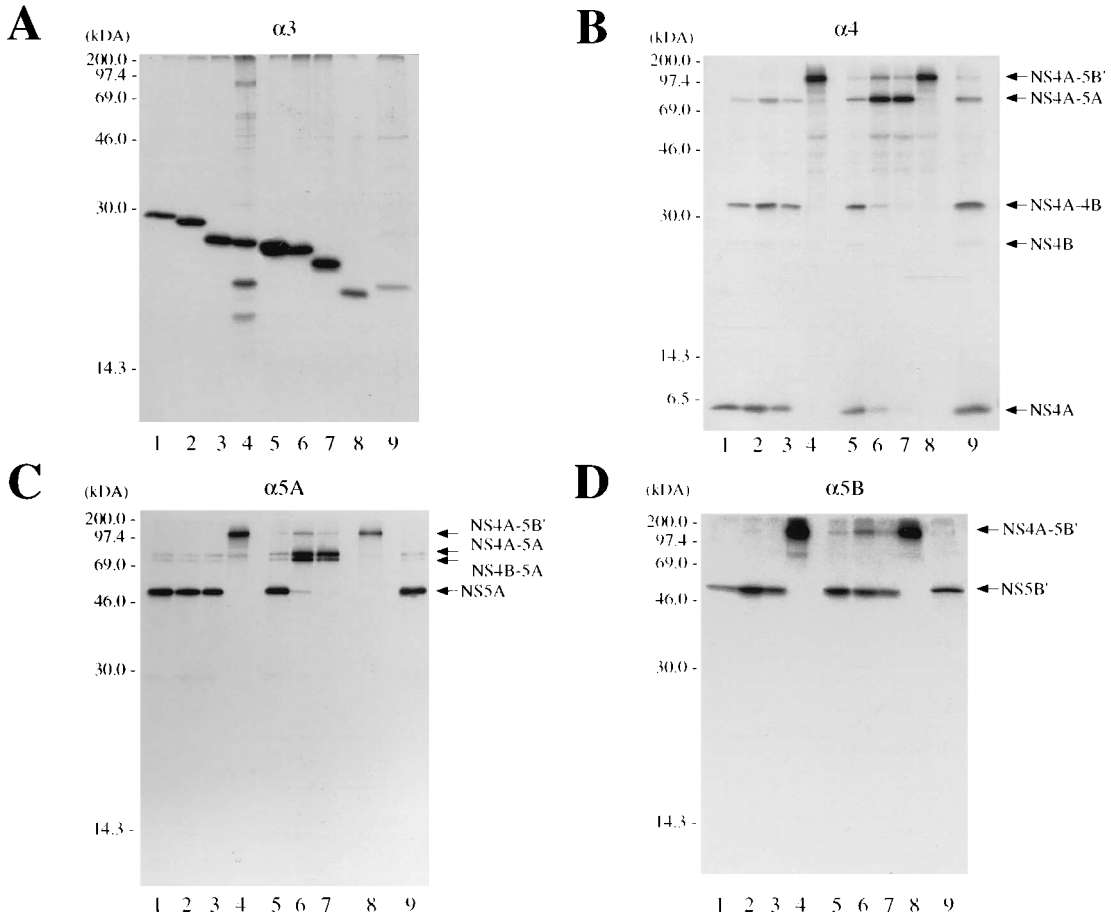

FIG. 4. Determination of the minimal serine protease domain of NS3 required for full processing activity. Cells were cotransfected with plasmid pCite(NS4-5) and one of the constructs encoding the different NS3 mutants: lanes 1, pCite(NS3[1007-1237]); lanes 2, pCite(NS3[1007-1216]); lanes 3, pCite(NS3[1007-1206]); lanes 4, pCite(NS3Flag[1015-1196]); lanes 5, pCite(NS3[1037-1237]); lanes 6, pCite(NS3[1042-1237]); lanes 7, pCite(NS3[1055-1237]); lanes 8, pCite(NS3[1076-1237]); lanes 9, pCite(NS3[1038-1206]). Four hours after transfection, the cells were labelled with [^{35}S]methionine for 1 h. Cell lysates were prepared and immunoprecipitated with rabbit antisera specific for NS3 (A, lanes 1 to 3 and 5 to 9), NS4 (B), NS5A (C), and NS5B (D). The 23-kDa NS3 derivative encoded by pCite(NS3Flag[1015-1196]) was immunoprecipitated by an immobilized antibody directed against the Flag sequence (A, lane 4). Immunoprecipitated proteins were then separated by SDS–13.5% polyacrylamide gel electrophoresis. HCV-specific proteins are indicated by arrows on the right. The sizes of molecular weight marker proteins are indicated on the left.

tate the corresponding protein with an anti-Flag antibody (see Materials and Methods). Control experiments have shown that the presence of this N-terminal Flag sequence does not interfere with the protease function when fused to the intact protease domain, as in pCite(NS3Flag[1015-1206] (data not shown). All of the mutated proteins were found to be synthesized in the cytoplasm of transfected cells, although the amounts of the individual mutant proteins detectable by immunoprecipitation were considerably different (Fig. 4A). This could be due to differences in transfection efficiency, reactivity toward the antibodies, level of expression, or intrinsic stability of the individual proteins. Bartenschlager et al. recently reported that most NS3 deletion mutants had half-lives shorter than those of full-length or nearly full-length NS3 molecules when expressed via a recombinant vaccinia virus (4).

We tested the activity of each deletion mutant by cotransfection of the corresponding plasmid with pCite(NS4-5), which encodes a precursor that contains NS4A, NS4B, NS5A, and most of NS5B (NS5B′). Cells were harvested after a pulse-labelling of 1 h. All of the expected cleavage products from the precursor were identified by immunoprecipitations of extracts obtained from cells expressing NS3 mutants NS3[1007-1237], NS3[1007-1216], and NS3[1007-1206] (Fig. 4B to D, lanes 1 to 3), indicating that the C-terminal portion of NS3 could be deleted up to amino acid 1206 without a detectable loss of activity. The further deletion of 10 amino acid residues (mutants NS3Flag[1015-1196]) led to a complete inactivation of the protease: immunoprecipitations with specific antisera evidenced only the uncleaved 140-kDa NS4A-5B′ precursor (Fig. 4B to D, lanes 4). Cotransfection of pCite(NS4-5) with pCite(NS3[1007-1196]) showed a similar phenotype, although we could not detect the production of the NS3[1007-1196] mutant protein (see above).

Analysis of the N-terminal deletion mutants of NS3 revealed a gradual inactivation of the protease. Normal cleavage products were immunoprecipitated from extracts of cells transfected with the NS3[1037-1237] (Fig. 4B to D, lanes 5), while only an uncleaved 140-kDa polypeptide coexpressing the NS4A-5B′ precursor was obtained with the NS3[1076-1237] mutant (Fig. 4B to D, lanes 8). NS3 proteins with intermediate deletions, NS3[1042-1237] and NS3[1055-1237], showed partially defective protease activity. Cotransfection of either pCite (NS3[1042-1237]) or pCite(NS3[1055-1237]) with pCite (NS4-5) resulted in the normal production of the 58-kDa protein specifically recognized by anti-NS5B antiserum (Fig. 4D, lanes 6 and 7), indicating efficient and rapid processing at the

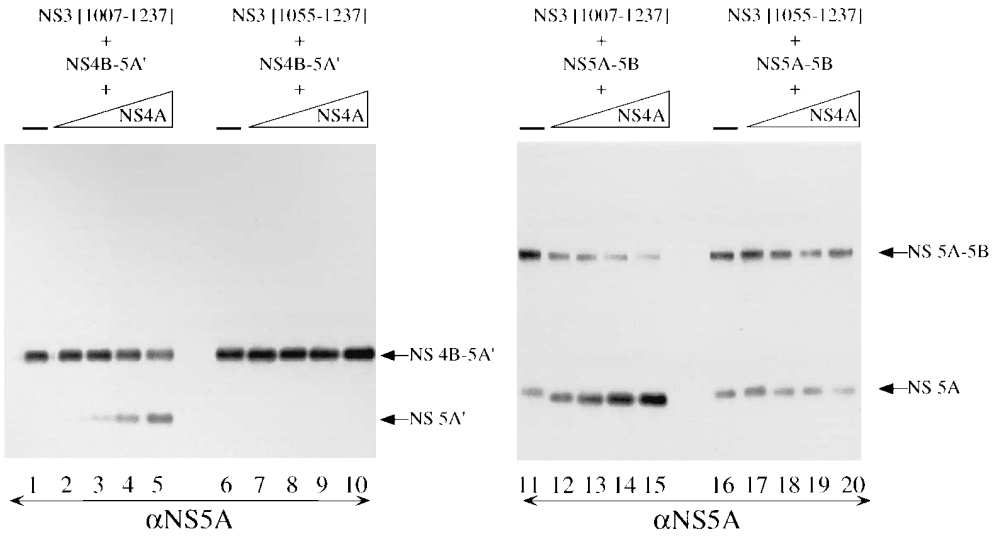

FIG. 5. NS3 protease activity in the presence of increasing amounts of NS4A. (A) Two micrograms of plasmid pCite(NS3[1007-1237]) (lanes 1 to 5) or pCite(NS3[1055-1237]) (lanes 6 to 10) was cotransfected with 10 μg of plasmid pCite(NS4B-5A) (lanes 1 and 6) or with 10 μg of plasmid pCite(NS4B-5A) and increasing quantities of pCite(NS4A): 0.5 μg (lanes 2 and 7), 2 μg (lanes 3 and 8), 5 μg (lanes 4 and 9), and 10 μg (lanes 5 and 10). (B) Two micrograms of plasmid pCite(NS3[1007-1237]) (lanes 1 to 5) or pCite(NS3[1055-1237]) (lanes 6 to 10) was cotransfected with 10 μg of plasmid pCite(NS5AB) (lanes 1 and 6) or with 10 μg of plasmid pCite(NS4B-5A) and increasing quantities of pCite(NS4A): 0.5 μg (lanes 2 and 7), 2 μg (lanes 3 and 8), 5 μg (lanes 4 and 9), and 10 μg (lanes 5 and 10). Four hours after transfection, the cells were labelled with [^{35}S]methionine for 1 h. Cell lysates were prepared and immunoprecipitated with an anti-NS5A rabbit antiserum. Immunoprecipitated proteins were separated by SDS–10% polyacrylamide gel electrophoresis and are indicated by arrows on the right.

NS5A/5B junction. In contrast, processing at the NS4A/4B site by these mutants occurred at lower efficiency, as judged by the diminished intensity of the protein band corresponding to NS4A (Fig. 4B, lanes 6 and 7). In addition, cleavage at the NS4B/5A site was greatly diminished, which resulted in the accumulation the NS4B-NS5A and NS4A-NS5A precursors (Fig. 4B and C, lanes 6 and 7). These results show that N-terminal deletions of NS3 produce differential effects on processing at different cleavage sites; in particular, the cleavage at the NS4B/5A junction is the most sensitive to the deletion of the NS3 N-terminal portion, whereas the cleavage between NS5A and NS5B seems to be relatively unaffected by the same mutation.

Analysis of the activity of the N-terminal and C-terminal deletion mutants of NS3 would indicate the region comprised between amino acids 1037 and 1206 of the HCV-BK polyprotein as necessary for full proteolytic activity at all *trans*-cleavage sites. To confirm that this region is not only necessary but also sufficient for the activity just described, we constructed plasmid pCite[NS3(1038-1206)] and analyzed the *trans*-cleavage activity of its protein product. As shown in Fig. 4B to D, lanes 9, the 169-amino-acid polypeptide encoded by pCite [NS3(1038-1206)] is fully competent for all cleavages contained in the NS4A-NS5B′ precursor.

The NS3[1055-1237] mutant does not interact with NS4A. The cleavage efficiency of NS3 is affected by NS4A differently on each cleavage site. The NS4B/5A junction is not cleaved at all by NS3 in the absence of NS4A, while the cleavage between NS5A and NS5B is accelerated only by NS4A (14). The finding that the NS3[1055-1237] mutant is in fact able to cleave efficiently only at the NS5A/5B junction suggests that an altered interaction with NS4A might be responsible for the partially defective cleavage activity of this mutant. To test this hypothesis, we carried out the experiments described below.

As expected, when we transfected pCite(NS4B-5A) with pCite(NS3[1007-1237]), we found no evidence of proteolytic cleavage (Fig. 5, lane 1). When NS4B-5A′ and NS3 were expressed in combination with increasing amounts of NS4A, the mature 46-kDa NS5A-related product was immunoprecipitated with the anti-NS5A antiserum (Fig. 5, lanes 2 to 5). The extent of cleavage was dose dependent with respect to NS4A. Immunoprecipitations of extracts from cells expressing the NS4A-NS5B′ substrate, the NS3[1055-1237] mutant, and increasing amounts of NS4A revealed only the 68-kDa precursor as if the proteolytic activity were completely abolished (Fig. 5, lanes 6 to 10).

Next, we transfected plasmid pCite(NS5AB) with pCite(NS3) and increasing amounts of pCite(NS4A). Immunoprecipitations of the cell extracts with the anti-NS5A antiserum showed a progressive increase in the production of mature NS5A protein (Fig. 5, lanes 11 to 15). In contrast, immunoprecipitations of cell extracts derived from cotransfection of pCite(NS5AB) with pCite(NS3[1055-1237]) and increasing amounts of pCite(NS4A) did not show any change in the product/precursor ratio (Fig. 5, lanes 16 to 20). This result indicates that NS3[1055-1237], which lacks the N-terminal 28 amino acids of NS3, retains basal protease activity at the NS5A/5B site but can no longer be activated by NS4A.

Our finding that deletion of the 28 N-terminal amino acids of NS3 impairs the proteolytic processing activity associated with this protein by preventing its activation by NS4A suggests that this region of NS3 might be involved in the formation of an NS3-NS4A complex. We studied the physical association of NS4A with either NS3[1007-1237] or NS3[1055-1237] in transfected cells by coimmunoprecipitation experiments under nondenaturing conditions. Figure 6A illustrates the result of immunoprecipitation carried out under denaturing (lanes 1 to 3) or nondenaturing (lanes 4 and 5) conditions, using samples generated by cotransfecting equivalent amounts of pCite (NS3[1007-1237]) with pCite(NS4A) and pCite(NS4B-5A). As expected, coexpression of NS3[1007-1237] (Fig. 6A, lane 1) and NS4A (Fig. 6A, lane 2) resulted in the cleavage of the NS4B-5A′ precursor (Fig. 6A, lane 3). Under nondenaturing conditions, NS3[1007-1237] and NS4A were efficiently coim-

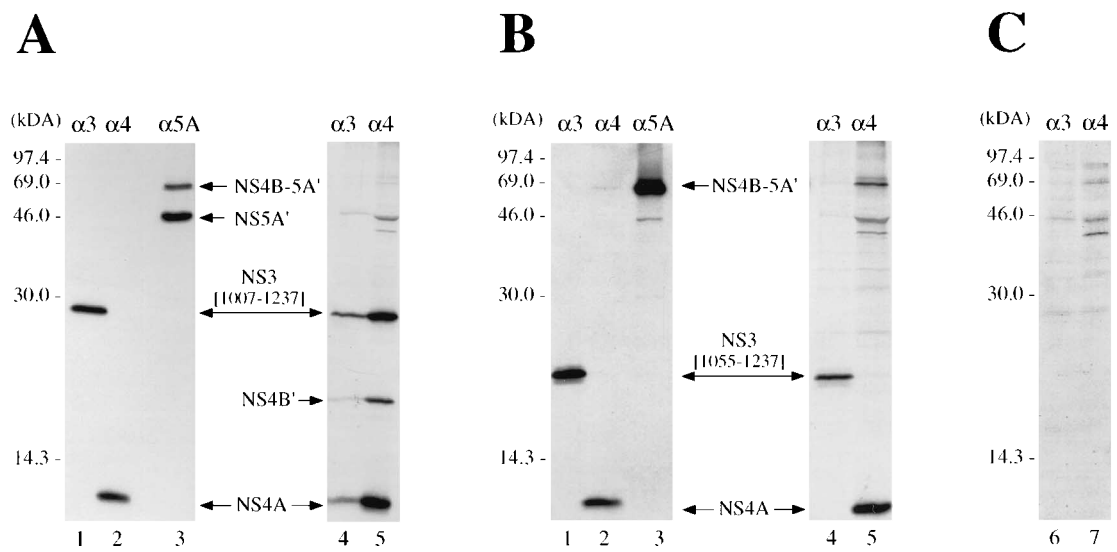

FIG. 6. Complex formation between the protease domains of NS3 and NS4A. Ten micrograms of plasmid pCite(NS3[1007-1237]) (A) or pCite(NS3[1055-1237]) (B) was cotransfected with 10 μg each of plasmids pCite(NS4B-5A) and pCite(NS4A). Four hours after transfection, the cells were labelled with [^{35}S]methionine for 1 h. Cell lysates were prepared under denaturing (lanes 1 to 3) or nondenaturing (lanes 4 and 5) conditions and immunoprecipitated with an anti-NS3 (lanes 1 and 4), anti-NS4A (lanes 2 and 5), or anti-NS5A (lanes 3) rabbit antiserum. (C) Mock-transfected cell lysates prepared under nondenaturing conditions and immunoprecipitated with an anti-NS3 (lane 6) or anti-NS4A (lane 7) rabbit antiserum. Immunoprecipitated proteins were separated by SDS–13.5% polyacrylamide gel electrophoresis. HCV-specific proteins are indicated by arrows on the right. The sizes of molecular weight marker proteins are indicated on the left.

munoprecipitated by both anti-NS3 and anti-NS4 antisera (Fig. 6A, lanes 4 and 5), indicating the presence of a stable complex between the two proteins. Interestingly, a third protein corresponding in molecular weight to the NS4B fragment liberated from the NS4B-NS5A′ substrate (NS4B′; Fig. 6A, lanes 4 and 5) was also coimmuniprecipitated with NS3[1007-1237] and NS4A, suggesting that NS4B may be engaged in the formation of a ternary complex with the former two proteins. Further experiments are needed to address this possibility.

Coexpression of NS3[1055-1237] (Fig. 6B, lane 1) and NS4A (Fig. 6B, lane 2) resulted in very little cleavage of the NS4B-5A′ precursor (Fig. 6B, lane 3). The apparent discrepancy between this finding and the complete lack of cleavage activity on the NS4B-NS5A′ precursor displayed previously by the same mutant even in the presence of NS4A (Fig. 5, lane 10) has to be related to the fact that 2 μg of each of the plasmids encoding the different NS3 derivatives was used for the experiment shown in Fig. 5, whereas in this experiment, we transfected 10 μg of the same plasmids in order to facilitate the formation of a complex. As shown in lanes 4 and 5 of Fig. 6B, neither the anti-NS3 or the anti-NS4 antiserum coimmunoprecipitated the two proteins under nondenaturing conditions. The results just described suggest that the same mutations of NS3 that impair *trans*-cleavage activity on those sites that require NS4A for efficient processing also weaken association of the serine protease domain with NS4A.

Proteolytic activity of the NS3 lacking the N-terminal 28 amino acids on the NS3/4A intramolecular site. In a previous report, we showed that the NS3/4A site is cleaved intramolecularly (in *cis*) by the NS3 protease and that cleavage at that site requires, in addition to an active serine protease domain, the presence of NS4A (14). It was therefore of interest to assess how the N-terminal deletion present in the NS3[1055-1237] mutant affected the proteolytic activity of NS3 at this intramolecular cleavage site. To this end, we constructed plasmid pCite(NS3[ΔN1055]-4A) (Fig. 1). This plasmid drives the expression of a deletion product of NS3 lacking the N-terminal 28 amino acids in the context of a precursor polypeptide that contains also NS4A. The intra- and intermolecular proteolytic activities of this precursor were then compared with those of an NS3-NS4A precursor bearing the intact N terminus of NS3. This latter polypeptide was encoded by plasmid pCite(NS3-4A) (Fig. 1). To study the intra- and intermolecular proteolytic activities associated with the two precursors, either pCite(NS3-4A) or pCite(NS3[ΔN1055]-4A) was cotransfected with pCite (NS4B-5A). Coexpression of pCite(NS3-4A) with pCite(NS4B-5A) resulted in the production of mature NS3 and NS4A (Fig. 7A, lanes 1 and 2, respectively). In addition, we could observe the occurrence of a partial cleavage of the 68-kDa NS4B-NS5A precursor to yield the mature 46-kDa NS5A-related product (Fig. 7A, lane 3). This result was expected on the basis of our previous observations (14). We studied the physical association of NS4A with NS3 by coimmunoprecipitation experiments under nondenaturing conditions and found that the anti-NS3 antiserum immunoprecipitated a protein band corresponding in size to mature NS3 (Fig. 7B, lane 1). In addition, a radioactive band corresponding in size to NS4A, visible at the bottom of the gel, was coprecipitated. Anti-NS4 antiserum, in addition to mature NS4A and a little uncleaved precursor, coimmunoprecipitated mature NS3 nearly quantitatively (Fig. 7B, lane 2).

Interestingly, when pCite(NS3[ΔN1055]-4A) was used in a similar cotransfection experiment, we could detect mature NS4A as well as the NS3-related product expected from intramolecular cleavage at the NS3/4A site (Fig. 7A, lanes 4 and 5, respectively). In contrast, immunoprecipitation with the anti-NS5A antiserum revealed only the presence of uncleaved 68-kDa NS4B-5A′ precursor (Fig. 7A, lane 6). These results indicate that the cleavage at the NS3/4A intramolecular site occurred despite the deletion of the N-terminal portion of the serine protease domain, but once released from the precursor, the N-terminally deleted NS3 protein was unable to cleave the NS4B-NS5A precursor. Under nondenaturing conditions, the anti-NS3 antiserum immunoprecipitated a protein band corresponding in size to the N-terminal deletion product of NS3 (NS3[ΔN1055]) and some unprocessed precursor (Fig. 7B,

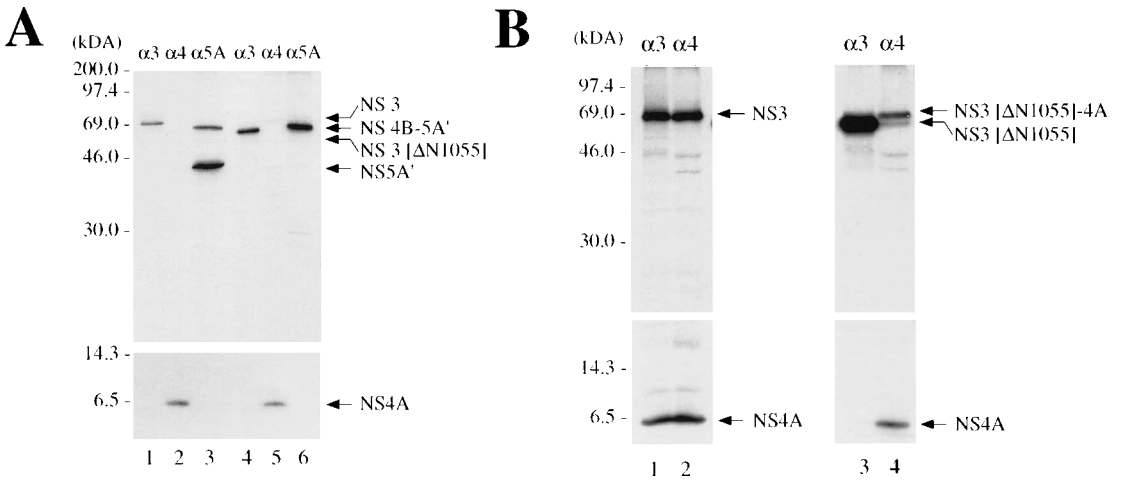

FIG. 7. (A) Effect of N-terminal deletion of NS3 on the *cis*-cleavage site. Ten micrograms of pCite(NS3-4A) (lanes 1 to 3) or pCite(NS3[ΔN1055]-4A) (lanes 3 to 6) was cotransfected with 10 μg of plasmid pCite(NS4B-5A). Four hours after transfection, cells were labelled with [^{35}S]methionine for 1 h. Immunoprecipitated proteins were separated by SDS–13.5% polyacrylamide gel electrophoresis. HCV-specific proteins are indicated by arrows on the right. The sizes of molecular weight marker proteins are indicated on the left. The lower portion of the gels, containing the NS4A proteins, were exposed four times longer than upper part. Cell lysates prepared under denaturing conditions were immunoprecipitated with an anti-NS3 (lanes 1 and 4), anti-NS4A (lanes 2 and 5), or anti-NS5A (lanes 3 and 6) rabbit antiserum. (B) As above, but cells lysates were prepared under nondenaturing conditions and immunoprecipitated with an anti-NS3 (lanes 1 and 3) or anti-NS4A (lanes 2 and 4) antiserum.

lane 3). The anti-NS4 antiserum precipitated selectively NS4A and the NS3[ΔN1055]-4A precursor, but very little mature NS3[ΔN1055] was coimmunoprecipitated (Fig. 7B, lane 4), indicating that the ability of this N-terminal deletion product of NS3 to form a stable complex with NS4A was severely impaired.

DISCUSSION

Analysis of processing kinetics with a precursor containing the nonstructural proteins from NS3 to NS5B has revealed that cleavages at the nonstructural sites are all relatively rapid: mature NS3, NS4A, NS5A, and NS5B are detectable after a pulse-labelling as short as 5 min. NS4B accumulates at a lower rate and is visible only after 20 min, but the early appearance of NS4A suggests that this is due to inherent instability of the NS4B protein rather than to delayed processing at the NS4A/4B site. Consistent with our finding, Bartenschlager et al. (4) have recently shown that NS4B is short-lived, with a half-life of only about 15 min. Cleavage at the NS3/4A and NS5A/5B sites occur most rapidly and very efficiently, as judged by the absence of a protein precursor containing either NS3 or NS5B even after short pulse-labelling of the transfected cells. In contrast, the persistence of NS4A-NS5A, NS4B-NS5A, and NS4A-NS4B precursors suggests that cleavage at the NS4A/4B and NS4B/5A sites is less efficient. The absence of partial proteolysis products containing either NS3 or NS5B may be interpreted also as suggestive of a precise order of polyprotein processing, as if cleavage at the NS3/4A and NS5A/5B sites were absolutely required for processing to continue at the remaining sites. Further studies will be necessary in order to clarify whether HCV polyprotein processing proceeds through preferred pathways. Similar kinetics of processing were observed in the *trans*-cleavage reaction. This might have been expected, since the cleavage at the NS3/4A site is very efficient and rapid (see above).

We exploited the *trans*-cleavage reaction on the NS4A-NS5B′ substrate in order to characterize a number of C-terminal and N-terminal deletion mutants of NS3. We found that a polypeptide as short as 169 amino acids, from 1038 to 1206 in the polyprotein, is sufficient and required for efficient proteolytic cleavage at all intermolecular cleavage sites. Whereas deletion of further 10 amino acids from the C terminus of this polypeptide led to complete inactivation of the protease activity, elimination of as few as 4 amino acids from the N terminus (NS3[1042-1237] and NS3[1055-1237]) abolished cleavage at the NS4B/5A site and impaired processing at the NS4A/4B site but surprisingly affected the proteolysis at the NS5A/5B site very little. Similarly, N-terminal deletion mutants of HCV NS3 have been shown to have differential effects on different proteolytic sites in a recent study in which recombinant vaccinia viruses were used to drive the expression of HCV polyproteins (4).

We and others have previously demonstrated that, in addition to NS3, the NS4A protein is strictly required for cleavage at the NS4B/5A site (4, 14). Conversely, cleavage at the NS5A/5B site is less dependent on the presence of NS4A. Our finding that the NS3[1042-1237] and NS3[1055-1237] mutants display a selective loss of cleavage activity on the NS4B/5A site could therefore be explained if these mutants were no longer able to be activated by NS4A. The experiments shown in Fig. 5 demonstrate that the proteolytic activity of the NS3[1055-1237] mutant is indistinguishable from that displayed by a fully functional NS3 protein in the absence of NS4A. It is therefore likely that the global folding of the serine protease is not grossly altered in this N-terminal deletion mutant. What has been lost is the ability of interacting with the cofactor NS4A: whereas the wild-type NS3 protease is stimulated by increasing amounts of NS4A, the mutant activity remains at the basal level regardless of the presence of the cofactor.

The mechanism by which HCV NS4A activates the NS3 serine protease is not yet known. It has been suggested that NS4A is required to target NS3 to the membrane for maximal protease activity. However, unequivocal evidence for membrane targeting is not available. Alternatively, NS4A could act as a virus-encoded chaperon in aiding the correct folding of the protease domain (11). In this regard, it is of interest that several bacterial serine proteases, including α-lytic protease and subtilisin, possess propeptides that share several proper-

ties with HCV NS4A: they are necessary for protease activity, can activate the protease either in *cis* or in *trans*, and are cleaved off the protease domain by an intramolecular proteolytic reaction (24, 35, 38). These propeptides are required to assist the folding of their protease counterparts, and when folding has been completed, the propeptide becomes dispensable and no longer required for proteolytic activity (2, 38). A third possibility is that the two proteins associate in a complex to form an active heterodimeric protease. In the case of flavivirus, it has been clearly demonstrated that active NS3 serine protease is engaged in a protein-protein complex with NS2B, a possible functional homolog of HCV NS4A (1, 7). Formation of the NS2B-NS3 complex has been shown to be a prerequisite for the serine protease activity of flavivirus NS3 (7). We now present evidence that also in the case of HCV, NS3 forms a complex with NS4A in vivo. Furthermore, N-terminal deletion mutants of NS3 whose proteolytic activity is no longer modulated by NS4A seem to be impaired in the ability to establish protein-protein interactions with this small protein cofactor, suggesting that a hetero-oligomeric complex between NS3 and NS4A may be the active species.

It is interesting that an NS3 mutant carrying the deletion of the 28 N-terminal amino acids is still competent for the *cis*-cleavage activity at the NS3/4A site. This finding was unexpected since in a previous work we provided evidence that NS4A is also required for this latter cleavage (14). How can an NS3 mutant that no longer interacts with NS4A display a proteolytic activity that does require NS4A? One possibility is that the intramolecular cleavage at the NS3/4A site has different structural requirements compared with the other, intermolecular sites. In fact, we have found that this site is the most tolerant to mutations of the residues flanking the scissile bond (13a), suggesting that processing in *cis* is affected primarily by polyprotein folding and to a lesser extent by the sequence of the cleavage site. Similarly, folding properties of the NS3-NS4A precursor could help position NS4A in a productive fashion even in the precursor containing the N-terminally deleted NS3 serine protease domain. The mutated precursor may then undergo self-cleavage because the cofactor is covalently tethered to the protease, as if covalent linkage were partially compensating a defective protein-protein interaction surface. As a consequence of intramolecular cleavage, NS4A will not stay associated with the mutated protease, and therefore the N-terminally deleted protease, once released from the precursor, will not be able to cleave at the NS4B/5A site in *trans*. On the basis of the data currently available, we favor a model in which interaction of the protease domain with NS4A occurs prior to and is necessary for the NS3-NS4A cleavage. A stable complex is then formed that is competent for all remaining cleavage events. If the N-terminal portion is deleted from NS3, then the resulting protein will not form a stable complex with NS4A and will therefore be defective for the *trans*-proteolytic activities of NS3 that do require NS4A as a cofactor.

Summarizing, we have identified a 28-amino-acid region of the HCV NS3 protease that is required for the modulation of the serine protease activity by NS4A. This region, found at the N terminus of the NS3 protein, is necessary for NS4A-dependent proteolytic activity of NS3 but not for its basal, nonstimulated serine protease activity. It is therefore likely that mutations in this region do not affect the proper folding of the serine protease domain of NS3 but affect only the ability of the protein to interact with NS4A. Consistent with this interpretation, we demonstrated that an NS3 molecule that is lacking the first 28 amino acids from its N terminus has lost the ability to form stable complexes with NS4A. Whether this region is an autonomous protein-protein interaction domain, sufficient per se to account for complex formation, remains to be established. More comprehensive genetic, biochemical, and eventually high-resolution structural studies are needed to precisely define the site of interaction and elucidate the mechanism by which NS4A modulates the proteolytic activity of NS3.

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