The hepatitis C virus NS3 serine proteinase and NS4A cofactor: Establishment of a cell-free trans-processing assay

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ABSTRACT The hepatitis C virus RNA genome encodes a long polyprotein that is proteolytically processed into at least 10 products. The order of these cleavage products in the polyprotein is NH₂-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. A serine proteinase domain located in the N-terminal one-third of nonstructural protein NS3 mediates cleavage at four downstream sites (the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites). In addition to the proteinase catalytic domain, the NS4A protein is required for processing at the 4B/5A site but not at the 5A/5B site. These cleavage events are likely to be essential for virus replication, making the serine proteinase an attractive antiviral target. Here we describe an in vitro assay where the NS3-4A polyprotein, NS5, the serine proteinase domain (the N-terminal 181 residues of NS3), and the NS4A cofactor were produced by cell-free translation and tested for trans-processing of radiolabeled substrates. Polyprotein substrates, NS4A-4B or truncated NS5A-5B, were cleaved in trans by all forms of the proteinase, whereas NS4A was also required for NS4B-5A processing. Proteolysis was abolished by substitution mutations previously shown to inactivate the proteinase or block cleavage at specific sites in vivo. Furthermore, an N-terminal sequence analysis established that cleavage in vitro occurred at the authentic 4A/4B site. Translation in the presence of microsomal membranes enhanced processing for some, but not all, proteinase-substrate combinations. Trans-processing was both time and temperature dependent and was eliminated by treatment with a variety of detergents above their critical micelle concentrations. Among many common proteinase inhibitors tested, only high (millimolar) concentrations of serine proteinase inhibitors tosyllysyl chloromethyl ketone and 4-(2-aminoethyl)benzenesulfonyl fluoride inactivated the NS3 proteinase. This in vitro assay should facilitate purification and further characterization of the viral serine proteinase and identification of molecules which selectively inhibit its activity.

Hepatitis C viruses (HCVs) have been recently identified as agents of the parenterally transmitted form of non-A non-B hepatitis (1, 2). Blood screening has virtually eliminated HCV-contaminated samples and greatly reduced the incidence of post-transfusion hepatitis. However, HCV remains responsible for a significant proportion of community-acquired hepatitis (3). Chronic infections are often established and can lead to chronic hepatitis and cirrhosis, which is strongly associated with development of hepatocellular carcinoma (reviewed in ref. 4). There is considerable interest in developing additional HCV-specific antiviral agents to complement currently available α-interferon therapy, which effectively controls disease in only a minority of HCV-infected patients.

The positive-strand HCV genome RNA is approximately 9.4 kb in length and consists of a highly conserved 5' noncoding region followed by a long open reading frame that encodes a polyprotein of ~3000 amino acids (reviewed in refs. 5 and 6). This polyprotein is cleaved at multiple sites by cellular and viral proteinases. The order and nomenclature of these cleavage products for the HCV-H strain are NH₂-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. C, E1, and E2 are putative structural proteins and the remaining proteins are presumed nonstructural (NS) proteins involved in RNA replication. The N-terminal domain of NS3 functions as a serine proteinase which cleaves at four sites in the NS region (at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites) (7–12). Substitutions for residues in the putative catalytic triad (such as Ser-1165) abolish processing at all four sites. While the NS3 serine proteinase domain alone is sufficient for cleavage at the 5A/5B site, coexpression of the S4-residue NS4A protein is required for processing at the 3/4A and 4B/5A sites, and perhaps also at the 4A/4B site (13–15). It has been suggested that the NS3 and NS4A proteins associate as a complex which is important not only for modulating substrate specificity of the serine proteinase (13–15) but also for membrane association of the HCV RNA replication complex (16).

Thus far, to our knowledge, in vitro assays useful for purification and further characterization of the NS3 serine proteinase and NS4A cofactor have not been described. In this report, we have established an in vitro trans-cleavage assay for these activities and have studied the effects of microsomal membranes, incubation temperature, detergents, and several common proteinase inhibitors.

MATERIALS AND METHODS

Cell-Free Transcription and Translation. HCV cDNA-containing expression constructs used in this study have been described (15). These plasmids (with the encoded proteins given in parentheses) include pTM3/HCV1027-1207 (NS3₁₁), pTM3/HCV1027-1657 (NS3), pBRTM/HCV1027-1711 (NS3-4A), pTM3/HCV1658-1711 (NS4A), pTM3/HCV1658-1972 (NS4A-4B), pTM3/HCV1712-2420 (NS4B-5A), and pTM3/HCV2269-2508 (NS5A₂₉₇₋₅B₈₈) (Fig. 1). Upstream (5') of the HCV cDNA sequences, all of these constructs contain a T7 promoter followed by the internal ribosome entry element of encephalomyocarditis virus (17). S'-Uncapped RNA transcripts were synthesized from linearized cDNA templates using T7 RNA polymerase (Epicenter Technologies, Madison, WI) (18). Cell-free translations using rabbit reticulocyte lysates (Promega) were performed according to the supplier's instructions. For 35S-labeled substrates, reaction mixtures were incubated for 60 min at 30°C in the presence of [35S]methionine (Amersham) at 300 µCi/ml (1 µCi = 37 kBq). To produce proteinase components, reaction mixtures were incubated for 90 min at 30°C with a mixture containing all 20 unlabeled amino acids (Promega). In some translation reactions, micro-

Abbreviations: HCV, hepatitis C virus; NS, nonstructural; TLCK, tosyllysyl chloromethyl ketone; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

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Fig. 1. HCV polyprotein, cleavage products, and expression constructs. The diagram of the HCV-H strain polyprotein indicates the positions and identities of the cleavage products including C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Sites of proteolytic processing are shown at the top, including those for host signal peptidase (●), the HCV NS2-3 autoprotease (●), and the HCV NS3 serine protease (●). The serine protease domain in the N-terminal one-third of NS3 is shaded. Shown below are the seven expression constructs used in this study. HCV polypeptide sequences present in each plasmid are indicated by black lines, which are drawn to scale and oriented with respect to the diagram of the HCV-H polyprotein. N- and C-terminal boundaries of truncated proteins are indicated by subscript numbers except for 3181, which denotes the N-terminal 181 residues of NS3. For simplicity, the NS prefix is not used here or in subsequent figures.

FIG. 1.

Results

An in Vitro Assay for HCV NS3 Serine Protease Activity. Various forms of the protease, with or without the NS4A cofactor, were produced by cell-free translation and tested for their ability to process radiolabeled substrates containing the 4A/4B, 4B/5A, or 5A/5B cleavage sites (see Fig. 1). We first tested the NS4B-5A polyprotein substrate, whose cleavage should be dependent upon expression of both the serine protease and the NS4A cofactor. The full-length NS4B-5A translation product (predicted molecular mass 76.5 kDa) was not processed after incubation with either the NS3 serine protease domain, NS3181 (Fig. 2A, lane 3), or the full-length NS3 protein (lane 5). When either form of the protease was produced by cotranslation with NS4A, proteolytic activity was observed as evidenced by the appearance of the NS4B and NS5A cleavage products (Fig. 2A, lanes 4 and 6). The putative NS5A cleavage product was difficult to observe in this assay, since it often comigrated with truncated translation products. NS4B, however, was readily observed and used as the major indicator of virus-specific proteolytic activity. When NS3 and NS4A were synthesized as a single polyprotein NS3-4A, significantly higher proteolytic activity was observed (Fig. 2A, lane 7). Due to the limited quantities of the cleavage products, we have not attempted N-terminal sequence analysis of NS5A. However, the sizes of the cleavage products, the requirement for both the NS3 serine protease and NS4A, and the immunoreactivity of the products (data not shown) suggest that authentic cleavage occurred in our in vitro assay. Further, substitution of an Ala residue for Ser-1165, the putative nucleophile of the NS3 serine protease (20), abolished...
processing in the in vitro assay, and processing was not observed for substrates containing mutations at the 4B/5A cleavage site that blocked cleavage in vivo (P1, Cys → Arg or Asp; ref. 21) (data not shown).

As mentioned earlier, the 5A/5B site can be cleaved by the NS3 proteinase in vivo in the absence of NS4A. Besides the full-length NS5A-5B polypeptide substrate, a truncated 240-residue polypeptide containing the 5A/5B cleavage site (NS5A_{297-5B88}) was also efficiently processed in trans by NS3_{181} in vivo (15). As shown in Fig. 2B, this substrate was processed inefficiently in the in vitro assay. Upon the addition of the various proteinases, we observed a slight but significant increase in the level of a 26-kDa species, tentatively identified as the truncated NS5A cleavage product (NS5A_{297-448}). It should be noted that this protein comigrated with a faint background band present in the original NS5A_{297-5B88} translation reaction (lane 2). The truncated NS5B cleavage product (NS5B_{1-88}), which is presumably unstable, has not been detected either in the in vitro assay or in vivo when the vaccinia virus T7 system has been used (15). In contrast to the NS4B-5A substrate, the appearance of the NS5A cleavage product was not markedly influenced by the presence of NS4A.

Translation and processing of the HCV polyprotein are believed to occur in the rough endoplasmic reticulum in association with microsomal membranes (6). Through their interaction with segments of the NS polypeptide rich in hydrophobic amino acids (either components of the proteinase or the substrates), microsomal membranes may also influence serine proteinase-dependent processing. We examined this possibility by studying the effects of microsomal membranes added to proteinase or substrate translation reactions. When the NS4B-5A substrate was synthesized in the presence of microsomes, the degree of processing by the various proteinases was unchanged (data not shown). Production of the NS4B and NS5A cleavage products was enhanced when NS4A was cotranslated with NS3_{181} or NS3 in the presence of microsomal membranes (Fig. 2A, lane 9 or 11). No stimulation was observed when microsomal membranes were added posttranslationally (data not shown). Interestingly, the proteolytic activity of NS3-4A was not increased by inclusion of microsomal membranes during translation (lane 12) (see Discussion).

Microsomal membranes might exert their influence on proteinase activity by interaction with NS3, NS4A, or both of these components. To examine this, transcripts encoding the serine proteinase domain, NS3_{181}, and NS4A were translated in separate reactions in the absence or presence of microsomal membranes and then combined and incubated with the NS4B-5A substrate. As shown in Fig. 2D, stimulation of proteinase activity correlated with the presence of microsomal membranes in the translation reaction mixtures of NS4A (lanes 4 and 6) but not NS3_{181} (lanes 6 and 7). These data suggest that nascent NS4A may cotranslationally interact with microsomal membranes in a way which stimulates serine proteinase activity, possibly by facilitating formation of an active proteinase complex between NS4A and the catalytic domain of NS3 (see Discussion). In contrast to our observations with the NS4B-5A substrate, microsomal membranes present during translation of the proteinase components did not enhance processing of NS5A_{297-5B88} (Fig. 2B). This is perhaps not surprising, given that this cleavage is not NS4A-dependent.

The most striking effect of microsomal membranes was seen for the NS4A-4B substrate. NS4A-4B synthesized in the absence of microsomal membranes was not processed by any of the proteinases (data not shown; also see Fig. 4). In contrast, NS4A-4B synthesized in the presence of microsomal membranes was efficiently processed by all sources of active serine proteinase, including NS3-4A, NS3, or NS3_{181} (Fig. 2C), but not the inactive proteinase containing the Ser-1165 → Ala substitution (data not shown). These results suggest that membrane association of NS4A-4B allows formation of an accessible 4A/4B cleavage site as well as a functional NS4A cofactor. N-terminal sequence analysis of the [35S]methionine-labeled NS4B cleavage product yielded methionine residues at positions 11 and 12 (data not shown), verifying site-specific cleavage at the authentic 4A/4B site (9) in vitro.

Further Characterization of the in Vitro Assay. A time course of NS3-4A-mediated processing of the NS4B-5A substrate is shown in Fig. 3A. At 30°C, a gradual increase in the amount of the NS4B and NS5A cleavage products was observed throughout the 3-h incubation period (Fig. 3A). Interestingly, after 3 h of incubation, the NS5A band shifted to a slower-migrating form, which is due to post-translational phosphorylation (K. Reed, A. Grakoui, J. Xu, C.L., and C.M.R., unpublished data). Compared with the rate at 30°C, NS4B-5A cleavage was slower at 23°C and faster at 37°C. No processing was observed at 0°C even after a 3-h incubation. Processing of NS4A-4B by the NS3_{181} proteinase (Fig. 3B) occurred rapidly even at 23°C, reaching a plateau after 15–30 min. Similar results were obtained at 30°C or 37°C (data not shown). Even at 0°C, a weak band corresponding to the NS4B cleavage product was observed after 2 or 3 h of incubation (Fig. 3B).

**FIG. 3.** Processing kinetics of NS4A-4B and NS4B-5A substrates. (A) Trans-cleavage reaction mixtures containing unlabeled NS3-4A proteinase and [35S]labeled NS4B-5A substrate (both synthesized in the absence of microsomal membranes) were incubated at 0°C, 23°C, 30°C, and 37°C. At the indicated time points, an equal portion of each trans-cleavage reaction mixture was analyzed by SDS/PAGE. (B) Trans-cleavage reaction mixtures containing unlabeled NS3_{181} proteinase (synthesized in the absence of microsomal membranes) and [35S]labeled NS4A-4B substrate (synthesized in the presence of microsomal membranes). Samples were processed and analyzed as in A. (C) Aliquots of NS3_{181} proteinase and [35S]labeled NS4A-4B substrate were preincubated for 30 min at the indicated temperatures and assayed for trans-processing by mixing and incubation for 1 h at 37°C. Samples not preincubated were used as positive controls (+). Products were resolved by SDS/PAGE.
To determine whether active proteinase or properly folded substrate was limiting in these trans-cleavage reactions, samples of NS3$^{181}$ or NS4A-4B were preincubated at various temperatures prior to the in vitro trans-cleavage assay. As shown in Fig. 3C, preincubation at 37°C for 30 min resulted in only slight inactivation of the proteinase (lane 4) or the substrate (lane 8). Both appeared to be stable at 0°C (lane 2) or 30°C (lanes 3 and 7). Higher temperatures resulted in increased inactivation of the proteinase (lanes 5 and 6) and, to a lesser extent, the substrate (lanes 9 and 10). NS3$^{181}$ activity was not detected after preincubation for 30 min at 50°C (lane 6).

Since the proteinase was reasonably stable at 30°C, these experiments suggest that the rate and extent of NS4A-4B cleavage may be limited by the amount of substrate in the correct conformation for efficient processing. Since cleavable NS4A-4B was produced only when microsomal membranes were present during translation, we examined the effects of varying the concentration of microsomal membranes or NS4A-4B RNA transcript on cleavage efficiency of the substrate. Increasing the concentration of microsomal membranes led to increased accumulation of primary translation product and enhanced processing efficiency at the 4A/4B site (Fig. 4).

The highest concentration of microsomal membranes tested (1.8 µl per 25-µl reaction mixture), decreasing the input RNA transcript lowered the level of the NS4A-4B translation product but did not significantly alter its cleavage efficiency (data not shown).

We also examined the effects of several detergents. All of the detergents tested showed significant inhibition of trans-cleavage of NS4B-5A by the NS3-4A proteinase (Fig. 5). Least severe inhibition was observed for MEGA-8 [critical micelle concentration (cmc) of 58 mM], which at 0.5% (15.6 mM) only slightly decreased the proteolytic activity of NS3-4A. Proteolysis was diminished in the presence of 0.1% (3.4 mM) n-octyl glucoside (cmc of 20–25 mM) and was abolished by the addition of n-octyl glucoside to 0.5% (17.1 mM). For CHAPS and CHAPSO (cmc of 8 mM), 0.1% (1.6 mM) but not 0.5% (8.0 mM) allowed detectable processing of NS4B-5A. No cleavage was observed in the presence of 0.1% of any of the following detergents: n-dodecyl β-D-maltoside, Triton X-100, Triton X-114, and n-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (also called Zwittergent 3-12) (data not shown). It should be noted that this concentration (0.1%) is above or close to the cmc for each of these six detergents. Similar results were obtained for NS3$^{181}$-mediated trans-processing of NS4A-4B (data not shown). These results suggest an inverse correlation between formation of detergent micelles and proteinase activity of NS3$^{181}$ or NS3-4A.

The serine proteinase domain of HCV NS3 was identified on the basis of sequence homology with members of the trypsin-like serine proteinase superfamily (reviewed in ref. 20), and the importance of predicted catalytic residues was subsequently verified by site-directed mutagenesis (see ref. 6 and citations therein). The in vitro assay allowed us to test the effects of common proteinase inhibitors on trans-cleavage by the HCV serine proteinase. The serine proteinase inhibitors tosylsulfonyl chloromethyl ketone (TLCK) and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) at millimolar concentrations inhibited both the NS3$^{181}$ and NS3-4A proteinases in the trans-cleavage assays utilizing either the NS4A-4B or NS4B-5A substrates (Fig. 6). Slight inhibition was observed with phenylmethylsulfonyl fluoride (PMSF; 2 mM), tosylphenylalanyl chloromethyl ketone (TPCK; 0.2 mM), and EDTA (10 mM) (data not shown). Other proteinase inhibitors, even at the highest concentrations tested, had no detectable effect on trans-cleavage activity.

These included serine proteinase inhibitors α1-antichymotrypsin (2 µM), aprotinin (2 mg/ml), benzamidine (20 mM), chymostatin (2 mM), 3,4-dichloroisocoumarin (2 mM), leupeptin (2 mM; also a cysteine proteinase inhibitor), soybean trypsin inhibitor (0.2 mM), and the cysteine proteinase inhibitor E-64 (0.4 mM) (data not shown).

![Fig. 4](image)

**Fig. 4.** Concentration effect of microsomal membranes on processing of NS4A-4B by NS3$^{181}$. $^{35}$S-labeled NS4A-4B was produced by translation (transcript RNA at 50 µg/ml) in the absence or presence of increasing amounts of microsomal membranes (indicated above each lane in µl per 25-µl reaction mixture). NS4A-4B substrates were incubated for 1 h at 37°C in the absence (Upper) or presence (Lower) of unlabeled NS3$^{181}$ (produced by translation in the absence of microsomes). Samples were analyzed by SDS/PAGE.

![Fig. 5](image)

**Fig. 5.** Effects of detergents on trans-processing by NS3-4A. NS3-4A proteinase and $^{35}$S-labeled NS4B-5A substrate were produced by translation in the absence of microsomal membranes. After translation, reaction mixtures were mixed in a 1:1 ratio and detergents were added to the final concentrations shown above each lane. MEGA-8, octanoyl-N-methylglucamide; n-OG, n-octyl glucoside; CHAPS, 3-[n-cholamidopropyl]dimethylammonio]-1-propanesulfonate; CHAPSO, 2-hydroxy-CHAPS. Trans-cleavage reaction mixtures were incubated for 3 h at 30°C and analyzed by SDS/PAGE.

![Fig. 6](image)

**Fig. 6.** Effects of proteinase inhibitors on the activity of the NS3$^{181}$ and NS3-4A proteinases. Unlabeled proteinases included NS3$^{181}$ (A) or NS3-4A (B and C). $^{35}$S-labeled substrates included NS4A-4B (A and B) or NS4B-5A (C). The NS4A-4B substrate was translated in the presence of microsomal membranes. The first lane in each set (−) is a solvent control containing no inhibitor (2 mM sodium acetate, pH 5.2, for TLCK; 4 mM sodium phosphate, pH 7.0, for AEBSF). Inhibitor concentrations (increasing from left to right) were: TLCK (2 µM, 20 µM, 200 µM, or 2 mM); AEBSF (20 µM, 200 µM, or 200 µM). Trans-cleavage reaction products were analyzed by SDS/PAGE.
DISCUSSION

In this report, cell-free translation was used to develop a trans-cleavage assay for the HCV serine protease. This approach has proven valuable for studying proteinases encoded by many animal and plant viruses. For HCV, proteolytic activity was obtained by expression of an NS3-4A polyprotein, full-length NS3, or the serine proteinase domain (NS3181). Active NS4A cofactor could be expressed as a cis-cleavage product of the NS3-4A polyprotein, as an individual polypeptide, or as part of the NS4A-4B substrate. Thus far, the requirements for trans-cleavage in vitro generally mimic those previously observed in transient expression experiments in mammalian cell cultures (13-15). Both the NS3 serine proteinase and the NS4A cofactor were required for trans-cleavage at the 4B/5A site, whereas the serine proteinase domain alone was sufficient for cleavage of NS4A-4B or a truncated substrate containing the 5A/5B site.

The role of NS4A in facilitating cleavage at certain sites in the NS polyprotein is still unclear. Of many possibilities, NS4A might help in the formation of an active proteinase by acting as a chaperone to facilitate proper folding, by interacting with the catalytic subunit in a way which facilitates substrate recognition and cleavage at NS4A-dependent sites, or by localizing NS3 to cellular membranes (16) where proteolysis of membrane-associated substrates might occur more efficiently (13-15). Our results (this report; ref. 22) argue against an obligate chaperone-like role, since similar trans-cleavage activity at the 5A/5B site was observed for all forms of the protease, regardless of NS4A coexpression. Common preproreport studies have provided evidence for an NS3-NS4A complex which may be responsible for cleavage at NS4A-dependent cleavage sites (22). Interestingly, we found that translation of NS4A in the presence of microsomal membranes enhanced proteolytic activity. Similarly, microsomal membranes were required to produce an NS4A-4B substrate which could be processed in trans. These results suggest that the nascent NS4A region, perhaps by means of its N-terminal hydrophobic domain, interacts with membranes to allow formation of an active proteinase complex, attain proper substrate conformation (as for NS4A-4B), or both. Microsomal membranes were not absolutely required to generate active NS4A cofactor and did not enhance the activity of the NS3-4A proteinase. Thus, while membrane association of the NS3-NS4A complex may be important for replicase function, it is not absolutely required for cleavage at NS4A-dependent sites.

For a variety of detergents, we found that trans-cleavage activity was inhibited or abolished as the detergent concentrations approached or exceeded their critical micelle concentrations. This observation contrasts with the flavivirus NS2B-NS3 serine proteinase complex, where activity is recovered only after solubilization of cellular membrane fractions with nonionic detergents (23). Computer modeling (24) and the results of mutagenesis studies (21) indicate that the HCV NS3 proteinase has a rather hydrophobic substrate binding pocket which might be capable of binding detergents or detergent micelles, leading to inhibition of proteinase activity. Alternatively, detergent micelles could exert their inhibitory effect by binding to NS4A or its cognate binding site on the NS3 proteinase domain. The NS3-NS4A complex, although not disrupted by treatment with nonionic detergents, appears to be stabilized by multiple hydrophobic interactions involving residues in the N-terminal and central regions of NS4A (22). Inhibition could be mediated either by changing proteinase conformation and specificity or by sterically hindering substrate access. Further studies are needed to distinguish among these possibilities.

Sequence alignments with known proteinases (see ref. 20 for a review), structural modeling of the active site (24), and mutagenesis studies of the proposed catalytic triad residues (7-12) are all consistent with the assignment of the HCV proteinase to the trypsin/chymotrypsin-like superfamily. This hypothesis is further supported by our finding that only serine proteinase inhibitors, in particular TLCK and AEBSF, markedly inhibited the HCV proteinase trans-cleavage activity. Millimolar inhibitor concentrations were needed for complete inhibition, suggesting that these common trypsin (TLCK) or general (AEBSF) serine proteinase inhibitors are not particularly effective for the HCV enzyme. Only slight inhibition was observed with other serine proteinase inhibitors, phenylmethylsulfonyl fluoride and tosylphenylalanyl chloromethyl ketone, or the metal ion chelator EDTA. The latter observation suggests that metal ions may somehow be involved in HCV serine proteinase activity. In any case, the establishment of an in vitro trans-cleavage assay and the preliminary results with these inhibitors are promising for future development of specific inhibitors of the HCV serine proteinase.

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