Inhibition of Hepatitis C Virus NS2/3 Processing by NS4A Peptides

IMPLICATIONS FOR CONTROL OF VIRAL PROCESSING*

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The NS2/3 protease of hepatitis C virus is responsible for a single cleavage in the viral polyprotein between the nonstructural proteins NS2 and NS3. The minimal protein region necessary to catalyze this cleavage includes most of NS2 and the N-terminal one-third of NS3. Autocleavage reactions using NS2/3 protein translated in vitro are used here to investigate the inhibitory potential of peptides likely to affect the reaction. Peptides representing the cleaved sequence have no effect upon reaction rates, and the reaction rate is insensitive to dilution. Both results are consistent with prior suggestions that the NS2/3 cleavage is an intramolecular reaction. Surprisingly, peptides containing the 12-amino acid region of NS4A responsible for binding to NS3 inhibit the NS2/3 reaction with Ki values as low as 3 μM. Unrelated peptide sequences of similar composition are not inhibitory, and neither are peptides containing incomplete segments of the NS4A region that binds to NS3. Inhibition of NS2/3 by NS4A peptides can be rationalized from the organizing effect of NS4A on the N terminus of NS3 (the NS2/3 cleavage point) as suggested by the known three-dimensional structure of the NS3 protease domain (Yan, Y., Li, Y., Munshi, S., Sardana, V., Cole, J. L., Sardana, M., Steinkühler, C., Tomei, L., De Francesco, R., Kuo, L. C., and Chen, Z. (1998) Protein Sci. 7, 837–847). These findings may imply a sequential order to proteolytic maturation events in hepatitis C virus.

Hepatitis C virus (HCV) is a positive-strand RNA virus that is the major cause of non-A, non-B hepatitis (1, 2). The HCV genome encodes a single polyprotein of approximately 3000 amino acids containing the viral proteins in the following order: C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B. The NS proteins are thought to be nonstructural and are involved with the enzymatic functions of viral replication and processing of the viral polyprotein. Release of the individual proteins from the polyprotein precursor is mediated by both cellular and viral proteases (3–6) (see also reviews in Refs. 7–9). The proteolytic release of mature NS4A, NS4B, NS5A, and NS5B is catalyzed by the chymotrypsin-like serine protease contained within the N-terminal domain of NS3 (termed “NS3 protease”), whereas host cell proteases release C, E1, E2, and p7, creating the N terminus of NS2 at amino acid 810 (10, 11). The cleavage between amino acids 1026 and 1027 of the HCV open reading frame that separates NS2 from NS3 is dependent upon protein regions of both NS2 and NS3 flanking the cleaved site; this autocleaving moiety is termed the NS2/3 protease (4, 12). The cleavage is independent of the catalytic activity of the NS3 protease, as demonstrated with mutational studies (4, 13).

The NS2/3 cleavage reaction has been studied in bacterial, mammalian, and insect cells or following in vitro translation of the protein (4, 5, 13–16). The protein region essential for NS2/3 cleavage activity has been approximately mapped to amino acids 898–1207 of the HCV open reading frame (4, 13, 14). The catalytic mechanism of NS2/3 cleavage is unknown but speculated to be either a metalloprotease (18) or cysteine protease (19). Cleavage activity of in vitro translated NS2/3 is inhibited by EDTA, and activity is restored with metal ion re-addition (13, 16).

There are no reported sequence homologies of the NS2 protein with proteins of known structure, and its N terminus is believed to be a transmembrane polypeptide (14). The NS3 protease domain structure is known in its mature form, however. The NS3 N terminus formed by NS2/3 cleavage is markedly affected by association with the cofactor peptide, NS4A. The x-ray crystallographic structure of NS3 bound to a peptide containing NS4A amino acids 21–34 (amino acids 1672–1685 of the HCV polyprotein) reveals that NS3 N-terminal residues 2–9 interact directly with NS4A to compose one of eight strands in an antiparallel β-sheet extending through the NS3 protease (20, 21). In contrast, without NS4A, the N terminus of NS3 is poorly organized (22).

Considering the paucity of data regarding the biochemistry of the NS2/3 reaction, it is of interest to probe the effects of a panel of peptides that represent natural ligands of NS2/3 upon the NS2/3 reaction rate. These peptides include sequences containing the NS3 binding region of NS4A as well as sequences containing the NS2/3 cleavage site. Because the detailed investigation of the NS2/3 reaction has been hampered by the lack of assays using defined components, we have employed here the demonstrated technique of in vitro translation to produce active NS2/3. The unexpected result of this study is that NS4A is a potent inhibitor, whereas the reaction is unaffected by potentially competing cleavage-site peptides.

MATERIALS AND METHODS

DNA Constructions—Two DNA constructs were made for the synthesis of the HCV NS2/3 J strand RNA and its subsequent translation to proteins, which lack the N-terminal membrane binding region of NS2 but contain HCV residues 849–1240, referred to as 894–1240J and Mal849–1240J. Codons 849–1240 were amplified by polymerase chain reaction from pT7 (a generous gift from Dr. Nicola La Monica (14)). For 849–1240J the HCV sequence was cloned into pET3c (Novagen), and for Mal849–1240J the DNA was inserted into pETMalcH (a generous gift of Dr. B. Leiting (23)), to produce an open reading frame encoding the fusion protein Mal849–1240, which includes Escherichia coli maltose-binding protein His6-tag-HCV residues 849–1240 (23). DNA for pCITE 810–1615BK was a generous gift from Dr. Nicola La Monica. Upon transcription and translation, pCITE 810–1615BK produces HCV residues 810–1615 of the BK strain, all of NS2, and most of NS3 (810–1615BK). The translation of this protein has been described pre-
vously (16). The expected masses for the proteins produced from these constructs are: 849–1240J, 42 kDa (cleavage products NS2, 19.6 kDa and NS3, 22.4 kDa); Mal849–1240J, 84.5 kDa (cleavage products Mal-NS2, 62.1 kDa and NS3, 22.4 kDa); 810–1615, 91 kDa (cleavage products NS2, 24 kDa and NS3, 67 kDa).

Site-directed mutagenesis was performed with the Stratagene Quick Change method to generate nonprocessing mutants H952A and C993A in the expression constructs described above.

Peptides—Peptides were obtained by custom synthesis from Midwest Biotech (Fishers, IN) and were greater than 95% pure as judged from reverse-phase high pressure liquid chromatography. Effective molecular weights were obtained by quantitative amino acid analysis. All peptides were dissolved and diluted in MeSO, so that the final concentration of MeSO in every reaction was 5%.

In Vitro Transcription and Translation—Circular DNA plasmids were linearized with BLP1 (Bpu 1102) and purified with a Qiagen QiaEX II kit before transcription. RNA was transcribed with T7 RNA polymerase (Ambion Megascript kit), phenol/CHCl₃-extracted, and ethanol-precipitated. Translations were with Promega or Ambion in vitro rabbit reticulocyte lysate translation kits at 30 °C for 30–40 min using [³⁵S]methionine as a label (Amersham Pharmacia Biotech). Translation was then inhibited by the addition of cycloheximide (250 μM final concentration), and samples were immediately frozen on dry ice.

NS2/3 Autocleavage Reactions—Translated NS2/3 was thawed on ice and cleavage was initiated by incubation at 20 °C, either in the original translation mixture or following a 10-fold dilution into a 10,000 molecular weight filtrate of reticulocyte lysate produced with Amicon Microcon-10 filters. Samples taken at the times indicated were combined with SDS-gel sample buffer and frozen on dry ice. NS2/3 cleavage reactions with the 810–1615BK were performed with 1% Triton X-100 present, as described previously (16). At the completion of an experiment, frozen samples were placed in boiling water for 5 min, and radiolabeled proteins were separated by SDS-PAGE (14%). Prestained Novex molecular weight standards were used in estimation of molecular weights of the products. For peptide inhibition measurements, incubations were initiated by the addition of diluted lysate to a MeSO solution of peptide in a tube held at 20 °C.

The distribution of ³⁵S-labeled proteins on dried gels was determined with a PhosphorImager (Molecular Dynamics). Protein bands were quantified and expressed as a proportion of total signal in the gel lane so that variations in gel lane loading were normalized. The product NS2 from 810–1615BK was used to generate data shown for screening of peptides directed at sites of cleavage to test their migrativeness on gels in a region with less background than the higher molecular weight products and its ability to initiate the 810–1615BK reaction with Triton X-100 (14). The IC₅₀ values were determined by first expressing the product level found as a fraction of the no-inhibitor control product level and then fitting the following equation, fractional activity = a + b[1 + (c/k)ᵢ], to the data, where a is the minimal level of fractional activity (tending to 0), a + b is the maximal level (tending to 1), x is the concentration of inhibitor, c is the IC₅₀, and d is a slope coefficient.

RESULTS

NS2/3 Processing Reactions—Typical NS2/3 processing reactions are shown in Fig. 1. The reaction occurred on a time scale of minutes, with the rate and final extent of reaction varying somewhat with the sequence expressed. NS2/3 810–1615BK was cleaved to as much as 60% with a 3-h incubation, and the maltose-binding protein fusion, Mal849–1240J, to nearly 100%. In all constructs, the mutations H952A or C993A prevented the appearance of products, as reported previously (4, 13). Both Mal849–1240J and 810–1615BK were used in subsequent characterization of the NS2/3 reaction and inhibition. NS2/3 from 810–1615BK was produced as a single 90-kDa band that cleaved itself to products of 65 (NS3) and 25 kDa (NS2), close to the expected molecular weights (Fig. 1A). In addition, 810–1615BK did not begin cleavage until the addition to Triton X-100, as has been reported (16), thereby allowing reactions to be initiated at will without background cleavage products generated during the translation phase of the experiment. The translation products of Mal849–1240J had the expected precursor molecular weight of 80 kDa, but also a smaller protein of 67 kDa (data not shown), possibly because of internal initiation, thus complicating the use of this version of NS2/3 for quantification of processing rates and inhibitor potencies.

Dilution of the NS2/3 precursor 10-fold into water completely prevented the processing reaction (data not shown). Dilution into a 10,000 molecular weight filtrate of rabbit reticulocyte

![Fig. 1. Hepatitis C NS2/3 processing reactions as observed with SDS-PAGE.](http://www.jbc.org/)
Inhibition of NS2/3 Cleavage by NS4A

TABLE I

Inhibition of NS2/3 by peptides

| Peptide name | Sequence | Inhibition | IC₅₀[a] μM |
|--------------|----------|------------|------------|
| NS 2/3 cleavage site-derived peptides[b] | | | |
| 17 | EGQWRLL*APITAYS | 15 | |
| 18 | GRGLRLL*APITAYS | 12 | |
| 19 | EGQWRLL | 14 | |
| 20 | APITAYS | −9 | |
| 21 | GRGLRLL | 2 | |
| 4 | GWRLL*APITA | 20 | |
| 5 | APITA | 6 | |
| 6 | GWRLL | 16 | |
| NS4A-derived peptides[c] | | | |
| 33 | KGSSVIVGRILSGRK | 61 | 5.7 |
| 4ApepB | VRLGSISVIGVIRGKK | −17 | 137.0 |
| 37 | Ac—RGGSVVIVGRILSGRK | 66 | 3.4 |
| 38 | GGSVVIVGRILSGRK | 66 | |
| 57 | KGSSVIVGRILSGRAVIPPR—NH₂ | 95 | |
| 54 | KGSSVIVGRILSGRAVIPPRESLYQEFDE | 85 | |
| 66 | Ac—KGSSVIV—NH₂ | 8 | |
| 67 | Ac—AILILSGR | −12 | |
| 74 | Ac—RIILSGRK | −21 | |
| Unrelated peptides[b] | | | |
| CMV-1-39 | GVVNAS.Abu.RLATRR | 14 | |
| HSV-1-1 | HTYLQASEKFKM | 11 | |

[a] IC₅₀ values were determined as described under “Materials and Methods” and are an average of two determinations.
[b] Percentages of inhibition shown for cleavage site and unrelated peptides were obtained with a final peptide concentration of 1 mg/ml, which when expressed as a molar concentration corresponds to a minimum of 500 μM for the group.
[c] Percentages of inhibition shown for NS4A peptides were obtained with a final peptide concentration of 50 μM.

Inhibition of NS2/3 reactions with 810–1615BK were performed for 60 min, as described under “Materials and Methods.” The NS2/3 cleavage site-derived peptides 17 and 18 correspond to HCV amino acids 1020–1033, J strain and BK strain, respectively. Other cleavage site-derived peptides are smaller segments of 17 or 18. The asterisk indicates the cleavage point, as determined in the NS2/3 protein (4, 12). Peptide 33 represents NS4A residues 21–34 and has lysine residues appended to each end to enhance solubility. Peptide 4ApepB has the same amino acids as 33, but in a random order. Similar results were obtained with NS2/3 Mal849–1240J. Abu, L-α-aminobutyric acid.

DISCUSSION

Despite numerous reports of NS2/3 processing reactions in cells and in vitro translation systems, a defined assay system containing purified NS2/3 protein is yet to be described. Existing techniques with in vitro translated NS2/3 have been exploited here to study the effects of potential peptide ligands of NS2/3. In the experiments described, the NS2/3 concentration is extremely low, possibly as low as 1 nM based upon an estimated specific activity of the radiolabeled protein produced (data not shown). The finding that dilution has no effect on the proportion of NS2/3 cleaved within a given time is consistent with an intramolecular mechanism. In addition, the lack of significant inhibition by potentially competing substrates, that is, peptides representing the cleavage site, is also consistent with an intramolecular cleavage.⁴

NS4A is identified here as an inhibitor of NS2/3 processing. In judging the potency of the inhibition, it is most reasonable to consider the case of a mechanism of NS2/3 cleavage as depicted in Scheme 1, where the rate constant k determines the rate of product accumulation, and a simple association reaction of NS2/3 with inhibitor (I) is governed by an equilibrium constant, Kᵢ.

⁴ A “bimolecular” cleavage has been reported (27) wherein a wild-type, processed NS2 was found to complement a C993A mutant of NS23.
Inhibition of NS2/3 Cleavage by NS4A

The rate of product (P) formation is given by
\[
dP/dt = k_{-1}[\text{NS2/3}]^n, \quad \text{where } n = 1.\]

Additionally, according to Scheme 1, \(K = [I][\text{NS2/3}]/[\text{NS3}]/\text{NS2/3}^3\) and \(dP/dt\) by 50% (the IC50) is the K. It is of interest, therefore, to compare the K values found here, 3.4 and 5.7 \(\mu\)M for peptides 33 and 37, respectively, with the K values determined for a NS3:NS4A complex. It has been reported that with a NS4A peptide (KKKGSVVIVGRIILSGR-NH2) at 1 mM, the association with polynucleotides, complicating the analysis of NS4A peptide effects upon helicase activity. The overall structure of NS2/3 may be affected similarly by NS4A binding, with residues critical for cleavage not positioned for the reaction in the NS4-bound state. Specifically, the cleavage site may be rendered inaccessible by NS4A binding, as depicted in Fig. 2.

The NS2/3 cleavage site sequence and the residues essential for cleavage are absolutely conserved in sequences of isolated strains of HCV, so that NS2/3 is presumed to be essential for viral replication. It may seem odd, therefore, that an essential cofactor for NS3 protease activity, the NS4A, would inhibit another essential process. The available data can be reconciled by the hypothesis that NS2/3 cleavage normally precedes NS4A release and NS3 protease processing of the rest of the NS region. In the absence of methods for replication of HCV in cell culture, the definition of the temporal sequence of HCV proteolytic events has not been possible. Our data presented here suggest that NS4A must not be released prematurely, or the NS2/3 cleavage would be inhibited. Although this idea appears inconsistent with in vitro translation studies showing that NS3:NS4A cleavage can occur in polyproteins wherein NS2/3 cleavage has not occurred (25), the rates of NS4A release have not been determined in those studies. The possibility remains that the normal sequence of proteolytic events in HCV replication includes NS2/3 cleavage as preceding NS3 protease action. The proper timing of proteolytic events has been demonstrated to be essential for viral replication in other systems such as HIV (26).

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8 It has been reported that NS4A also inhibits NS3 helicase through its association with the NS3 protease domain (17, 28). We find that NS4A peptides bind strongly to polynucleotides, complicating the analysis of NS4A peptide effects upon helicase activity.
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