In Vitro Activity of Hepatitis C Virus Protease NS3 Purified from Recombinant Baculovirus-infected Sf9 Cells*

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A recombinant Baculovirus expression system was used for the production of a 20-kDa protein encompassing the hepatitis C virus NS3 protease domain. The protein was purified to apparent homogeneity after detergent extraction of cell homogenates. It was shown to be a monomer in solution and to cleave the N-terminal region of NS4A. The enzyme also cleaved a 20-mer peptide corresponding to the NS4A-NS4B junction with $k_{cat}/K_m = 174 M^{-1} s^{-1}$. Peptides harboring NS4A sequences comprising amino acids 21–54 (Pep4A21–54) and 21–34 (Pep4A21–34) were found to induce an up to 2.8-fold acceleration of cleavage. Kinetic analysis revealed that this acceleration was due to an increase in $k_{cat}$, whereas no significant effect on $K_m$ could be detected. Pep4A21–54 was also an absolute requirement for cleavage of in vitro translated NS4B-NS5A by the purified protease. From these data we conclude that: (i) the purified protease domain shows substrate specificity and cleavage requirements similar to those previously reported on the basis of transfection experiments, (ii) activation of the purified protease by the NS4A co-factor can be mimicked by synthetic peptide analogs, and (iii) a central hydrophobic region of NS4A with a minimum core of 14 amino acids is responsible for the interaction with NS3.

The hepatitis C virus (HCV) is the causative agent of parenterally transmitted non-A non-B hepatitis (1, 2). The virus contains a positive stranded RNA genome of 9.5 kilobases with a single open reading frame encoding for a polyprotein of 3010–3033 amino acids (3–6). Upon translation this polyprotein is proteolytically processed into nine different polypeptides, which are encoded as follows on the viral RNA: 5’-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3’.

The mature structural proteins, C (the nucleocapsid protein), E1, and E2 (the two envelope proteins) have been shown to arise via proteolytic processing by host signal peptidases (7, 8). Conversely, generation of the mature nonstructural proteins NS2 through NS5B relies on the activities of virally encoded proteases. Thus, cleavage at the NS2-NS3 junction is accomplished by an as yet poorly characterized protease encoded between NS2 and NS3 (9, 10). We (11) and others (12–16) have shown all subsequent cleavages downstream of NS3, i.e. at the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junctions, to be catalyzed by a serine protease contained within the N-terminal region of NS3.

Characterization of cleavage events has shown that there are kinetic differences in processing of the single junctions. Thus, while processing between NS3 and NS4A is an intramolecular event, cleavage at all other sites has been demonstrated to occur in trans.

Homology modeling of the active site of the NS3 protease has permitted us to predict the preference for a cysteine residue at the P1 (according to the nomenclature introduced by Schechter and Berger (17)) positions of the substrates (18). Subsequent sequencing of the single cleavage sites has partially confirmed our predictions and yielded the consensus sequence D/E-X-X-C-A/S for all trans cleavage sites, with X being any amino acid and the scissile bond being located between C and A or S (12, 18). Notably, the intramolecular cleavage site between NS3 and NS4A differs from this consensus having a threonine residue in the P1 position.

NS3 is necessary, but not sufficient, for cleavage events within the polyprotein between NS3 and NS5B. As a matter of fact we (19) and others (20) have shown the NS3 protease to be a heterodimeric protein in vivo consisting of both NS3 and NS4A. Truncation experiments have mapped the N terminus of NS3 as the domain responsible for interaction with NS4A (21). The same region has been recently shown to be sufficient for NS4A binding when fused to a heterologous protein (22). In transfection experiments the interaction between NS3 and NS4A accelerates basal cleavage rates at the NS4A-NS4B and NS4A-NS5B junctions, whereas no cleavage occurs at the NS4B-NS5A site in the absence of NS4A, making the correct processing of the NS4B-NS5A precursor completely dependent upon the presence of NS4A (19, 20).

NS4A is a 54-residue protein. Deletion mutagenesis experiments have demonstrated that a central region of NS4A spanning from residue 22 through 34 is sufficient for co-factor activity (23).

Neither the mechanism of protease activation by NS4A nor the reasons for the different cleavage kinetics are known. Mechanistic investigations are hampered by difficulties in obtaining sufficient amounts of pure, active protease. As a matter of fact, the NS3 protein is a multidomain protein of 70 kDa, which, in addition to the protease domain at the N terminus, contains a putative RNA-helicase at its C terminus. C- and N-terminal truncation experiments (21) have demonstrated that a 20-kDa N-terminal fragment of NS3 is capable of performing all cleavages in vitro translation and in transfection experiments with an efficiency indistinguishable from that of the wild type enzyme, retaining its ability to interact with NS4A (21).

In this paper, we report the expression of this NS3 protease domain in a Baculovirus expression system, the purification

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1 The abbreviations used are: HCV, hepatitis C virus; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Fmoc, 9-fluorenylmethoxy carbonyl; HPLC, high performance liquid chromatography; NS, nonstructural; TPCK, tosylphenylalanyl chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis.
HCV Protease

and the characterization of the in vitro activity of this protein. We further show that peptides encompassing sequences of NS4A are capable of activating the purified protease and demonstrated that a minimum central core region of 14 amino acids in fact mediates the interaction of NS4A with NS3.

MATERIALS AND METHODS

Construction of Recombinant Baculovirus—To construct the plasmid pBacPro, a DNA fragment spanning the NS3 protein from residue 1038 to residue 1226 of the HCV polyprotein was obtained by polymerase chain reaction using appropriate oligonucleotides, which insert an ATG codon followed by a TAG stop codon at the 3'-end of the sequence. The fragment was inserted into the BamHI site of the pBlueBacII vector which was previously filled with the Klenow enzyme. The donor fragment was completely sequenced in order to exclude the introduction of mutations by polymerase chain reaction.

The pBacNS5AB plasmid was obtained inserting into the BamHI site of the pBlueBacII vector a DNA fragment encoding HCV polyprotein from amino acids 1997 to 2011. Linearized AcNPV DNA (Invitrogen) was co-transfected with each plasmid into the insect cell line Sf9 to obtain recombinant Baculovirus vBacPro and vBacNS5AB expressing the NS3 protease domain or the NS5AB polyprotein, respectively. Viral plaques were isolated and amplified according to the protocol recommended by the manufacturer.

Peptide Immunoprecipitations—Sf9 cells seeded at a density of 2 × 10^6/cm² plate were co-infected with the recombinant Baculovirus vBacPro and vBacNS5AB at a multiplicity of 5 plaque-forming units/cell. At 24 h post-infection the medium was replaced with Grace's medium lacking methionine (Life Technologies, Inc.), and the cells were starved for 1 h at 27 °C. Cells were then radiolabeled for 4 h with 280 μCi of [35S]-ProMix (Amersham Corp.) in Grace's medium lacking methionine and supplemented with 2% dialyzed fetal calf serum. Cells were harvested and lysed for immunoprecipitation in 150 ml of IPB solution (0.5 ml Tris, 80 mM NaCl, 1% Triton). Immunoprecipitations on denatured extracts were performed as described (11).

Subcellular Fractionation and Sedimentation of Membrane-associated Proteins—Sucrose density gradients were prepared by layering 2 × 10^8 cells in 10 ml of buffer D (50 mM triethanolamine, pH 7.5, 250 mM sucrose, 3 mM MgCl₂ and 1 mM DTT). 50 Ci of [35S]-ProMix (Amersham Corp.) in Grace's medium lacking methionine was added to each sample, followed by centrifugation for 3 min at 1000 g. The post-nuclear fraction (0.5 ml) was loaded onto a 0.5 ml NheI-Sal, 2 column volumes of equilibration buffer, the protease was eluted with a linear 0–1M NaCl gradient. In this and in the subsequent chromatographic steps the presence of the NS3 protease in the single fractions was detected by silver staining as well as by Western blots of precast 12.5% polyacrylamide gels run under denaturing conditions (PhastSystem, Pharmacia). The pooled, NS3-containing fractions were concentrated to 3 ml using an Amicon stirred ultrafiltration cell equipment with a YM 10 membrane and chromatographed on a HR 26/60 Superdex 75 column (Pharmacia) equilibrated with 50 mM sodium phosphate buffer, pH 7.5, 1% glycerol, 2 mM DTT, 1 mM EDTA, 0.1% CHAPS, operating at a flow rate of 2 ml/min. After washing with 2 column volumes of equilibration buffer, the protease was eluted with a linear 0–1 mM NaCl gradient. In this and in the subsequent chromatographic steps the presence of the NS3 protease in the single fractions was detected by silver staining as well as by Western blots of precast 12.5% polyacrylamide gels run under denaturing conditions.

Peptide and HPLC Assays—Peptides were synthesized by solid phase synthesis based on Fmoc chemistry. After cleavage and deprotection the crude peptides were purified by HPLC to >98% purity. Identity of peptides was checked by mass spectrometry. Concentration of stock solutions of peptides, prepared in Me2SO and kept at −20 °C, was determined by ultraviolet absorption, pH 7.4, in 100 mM sodium phosphate buffer, pH 7.5. Peptides were diluted with 96% ethanol and 4% water, respectively, and 2 μM of each peptide was incubated for 1 h and 0.5 μl of the reaction was loaded onto a 12% SDS-PAGE gel, stained with Coomassie blue, and visualized by autoradiography.

Cleavage assays were performed using 300 nm to 1.6 μM enzyme in 30 μl of 50 mM Tris, pH 7.5, 50% glycerol, 2% CHAPS, 30 mM DTT, and 0.05% CHAPS operating at 0.5 ml/min. Elution was achieved by applying a 0–5 μl NaCl gradient. Protein conversion was determined from UV spectra by determination of the absorbance at 280 nm: an extinction coefficient of ε = 18,200 M⁻¹ cm⁻¹ was calculated on the basis of primary sequence data according to published procedures (24), and concentration of NS3 was determined by a modified version of the Lowry method (25) with bovine serum albumin as standard. The final yield was 0.5 μg of purified cell pellet from cultured cells.

In Vitro Translation of NS3 Substrates—Appropriate DNA fragments derived from HCV-BK (5) cDNA were inserted downstream of the 5'-untranslated region of encephalomyocarditis virus and under the T7 promoter in the pCite-1 vector (Novagen) in the appropriate transla- tion reading frame and followed by a termination codon.

The plasmids pCiteNS3-4A, pCiteNS4AB, pCiteNS5AB, and pCiteNS5AB expressing, respectively, the HCV proteins NS3–4A from residue 992 to residue 1711, NS4AB from residue 1649 to residue 2380, and NS5AB from residue 1965 to residue 3010 were described previously (19). NS5Apro was obtained from pCiteNS3-4A by digestion with Msci and SalI, resulting in a transcript expressed residues 1462–1711. NS5BNS5AΔC216 was obtained from pCiteNS5BΔ458 by digestion with NheI, resulting in a transcript encompassing residues 1712–2203, while NS5A-NS5BΔC51 was obtained by digestion of pCiteNS5AB with Msci and SalI. In vitro transcription was done with T7 RNA polymerase. The transcripts were translated for 1 h at 30 °C in the presence of [35S]methionine using an RNA-dependent rabbit reticulocyte lysate (Promega). Aliquots of purified NS3 protease were added to the translated proteins, and the mixture was incubated for up to 2 h at 22 °C. Cleavage of labeled precursors was assessed by SDS-PAGE on 12.5% gels. Control samples with no enzyme were included to verify the identity of precursors and cleavage products by immunoprecipitation using specific antisera as described previously (11). Data were analyzed on a Phosphorimager and quantified by volumetric integration using ImageQuant software.

Inhibition Studies—[35S]-Labeled NS5AΔC51 was produced by in vitro translation as described above. After 1 h at 30 °C, translation was stopped by addition of 2.5 mM cycloheximide. 1 μM protease in 50 mM sodium phosphate buffer, pH 7.5, 50% glycerol, 2% CHAPS, 30 mM DTT were preincubated with different protease inhibitors at 22 °C for 1 h, and 5 μl of these solutions were added to 15 μl of labeled substrate. After 2 h at 22 °C the reaction was stopped by addition of SDS sample buffer (26). Samples were run on a 12% SDS-PAGE, and bands were visualized by staining with 10% Coomassie blue.
appropriate amounts of substrate and/or NS4A-peptide, such that the final concentration of Me₂SO did not exceed 10%. This Me₂SO concentration was shown not to affect enzyme activity. After incubation for variable time intervals at 22 °C, the reaction was stopped by addition of 70 µl of H₂O containing 0.1% trifluoroacetic acid. pH dependence experiments were carried out using the following buffers: pH 6.0–7.5, sodium phosphate; pH 7.5–9.0, Tris; pH 9.0–10.5, sodium borate. At overlapping pH values activity was determined with two different buffer systems and shown not to be affected by buffer composition. Ionic strength was kept constant at 20 mM.

Cleavage of peptide substrates was assessed by HPLC using a Merck-Hitachi chromatograph equipped with an autosampler. 90-µl samples were injected on a reversed phase HPLC column (C18 LiChrospher, 5 µm, 0.4 × 12.5 cm, Merck) and fragments were separated using a 30–100% acetonitrile gradient at 2%/min. Peak detection was done by monitoring both absorbance at 220 nm and the fluorescence of the N-terminal F-moc group (excitation, 260 nm; emission, 305 nm). Peptide fragments eluting from the HPLC column were collected and identified by mass spectrometry.

Cleavage products were quantified by integration of chromatograms with respect to the standard peptide F-moc-Y-Q-E-F-D-E-M-E-E-C. Initial rates of cleavage were determined at <20% substrate conversion. The kinetic parameters of the proteolysis reaction were calculated from least squares fit of initial rates as a function of substrate concentration assuming Michaelis-Menten kinetics with the help of a Grafit or a Kaleidograph software.

Expression of enzymatically active, membrane bound NS3 protease domain in Sf9 cells. A. Sf9 cells were infected with recombinant Baculovirus as described under “Materials and Methods.” 1.3 × 10⁸ cells were collected by centrifugation at the indicated time points, lysed in SDS sample buffer, and loaded on a 15% polyacrylamide gel. The protease was visualized by immunoblotting. The migration positions of molecular mass markers are indicated on the left. B, homogenates of Sf9 cells expressing the NS3 protease were prepared in 20 mM HEPES, pH 8.0, 1.5 mM MgCl₂, 0.5 mM EDTA, 3 mM DTT and centrifuged for 1 h at 100,000 × g. Pellet (lane 1) and supernatant (lane 2) were loaded on a 15% polyacrylamide gel followed by an immunoblot using anti-NS3 antibodies. The pellet was subsequently loaded on a 38–65% sucrose gradient in 20 mM Tris, pH 7.5, 150 mM KCl, 2 mM MgCl₂ and centrifuged for 16 h at 100,000 × g. The presence of the protease in the single fractions was monitored by immunoblotting. The triangle indicates increasing sucrose concentration. C, Sf9 cells were co-infected with recombinant Baculoviruses encoding the NS3 protease and the NS5A-NS5B substrate as described under “Materials and Methods.” After labeling with ³⁵S, proteins were immunoprecipitated using anti-NS3 (α3), anti-NS5A (α5A), or anti-NS5B (α5B) antibodies. The immunoprecipitates were loaded on 12% polyacrylamide gels and revealed by autoradiography. The molecular masses were estimated from the migration positions of ¹⁴C-labeled molecular mass markers.

Expression of the NS3 Protease Domain in Recombinant Baculovirus-infected Sf9 Cells—Infection of Sf9 cells with a recombinant Baculovirus resulted in expression of immunoreactive NS3 protein with the expected molecular mass of 23 kDa (Fig. 1A). Time course experiments showed increasing amounts of protein to be expressed up to 72 h post-infection, after which a plateau level was reached (Fig. 1A).

Upon centrifugation at 100,000 × g, the protein was almost quantitatively detectable in the pellet (Fig. 1B, lanes S and P). To discriminate whether this was attributable to formation of protein aggregates or to a partitioning of the protein into cell membrane the pellet was loaded on sucrose gradients (Fig. 1B). During these centrifugation experiments the immunoreactive NS3 protein migrated at low density, demonstrating that the protease was not present under the form of an aggregate and was most likely membrane-bound in Sf9 cells.

To determine whether the protein was also expressed in an enzymatically active form Sf9 cells were co-infected with recombinant Baculovirus encoding the NS5A-NS5B precursor protein. Cells were labeled with ³⁵S)methionine, and proteins were immunoprecipitated with anti NS3, anti-NS5A, or anti-NS5B, respectively (Fig. 1C). The appearance of immunoprecipitable polypeptides, migrating at the expected molecular mass positions of mature NS5A and NS5B, indicated the expression of enzymatically active NS3 protease in Sf9 cells (Fig. 1C). In control cells not expressing NS3, on the other hand, only the uncleaved NS5A-NS5B precursor was detectable. These findings prompted us to attempt the purification of the enzyme.

Purification of the NS3 Protease Domain—As a first step toward the purification of the enzyme, we tested the capability of a series of non-ionic and zwitterionic detergents to solubilize the protease. To this purpose, we checked the amount of im-

\[ \frac{dV}{dV_{\text{max}}} = \frac{V_{\text{max}}}{E_0} \]  

\[ (\text{Eq. 1}) \]
munoreactive protein in 120,000 × g supernatants of detergent-treated cell extracts. Activity of the solubilized enzyme was measured by assaying the cleavage of in vitro translated NS5A-NS5B precursor protein (data not shown). In this experimental design the detergent CHAPS gave the best results and was therefore used as solubilizing agent as well as added to all buffers during the purification procedure, which is depicted in Fig. 2.

Sf9 cells expressing the NS3 protease were collected 72 h post-infection. After homogenization and centrifugation of the homogenate, the resulting 120,000 × g supernatant was chromatographed on a cation exchange column at pH 6.5. followed by gel filtration chromatography, which simultaneously shifted the pH of the sample to 7.5. A final ion exchange step performed at pH 7.5 removed residual contaminants and yielded a protein that was homogeneous as judged by SDS-PAGE (Fig. 2).

Since molecular mass determination under native and under denaturing conditions yielded the same results (molecular mass of 22.8 kDa as judged from SDS-PAGE, 23.6 kDa as determined by gel filtration, versus 20.1 kDa calculated from the primary sequence), we conclude that the protein was present as a monomeric species in solution.

Activity on in Vitro Translated Substrates—In order to characterize the enzymatic activity of the purified protease, we investigated its ability to cleave the HCV polyprotein precursor. To this purpose 35S-labeled precursor proteins corresponding to all cleavage sites, i.e. NS3-NS4AΔpro, NS4A-NS4B, NS4B-NS5AΔC216, and NS5A-NS5BΔC51 were synthesized by in vitro translation from the appropriate RNAs. In the case of the NS3-NS4A precursor, the protease domain was deleted to avoid self-cleavage, whereas C-terminal deletions were introduced into the NS4B-NS5A and NS5A-NS5B substrates to increase the stability of the precursors. Incubation with purified NS3 protease yielded mature cleavage products in a dose-dependent fashion when either NS4A-NS4B or NS5A-NS5BΔC51 were used as substrates (Fig. 3). However, no cleavage products could be detected in the case of the NS3-NS4AΔpro and NS4B-NS5AΔC216 precursors (Fig. 3). This result is in line with previously reported transfection experiments, since cleavage at the NS4B-NS5A site is absolutely dependent upon the presence of NS4A in vivo (19), and the NS3-NS4A cleavage site was shown to be processed exclusively intramolecularly.

We wanted to address the question of the role of NS4A in the cleavage efficiency of the protease at different sites and synthesized a 34-mer peptide corresponding to the C terminus of NS4A by solid phase synthesis (Pep4A21-54, see "Materials and Methods"). We compared the effects of this peptide on the activity of the protease on all precursors. As shown in Fig. 3, Pep4A21-54 caused a modest increase of processing at the NS4A-NS4B and NS5A-NS5B sites. The former precursor already contains the NS4A sequences present in Pep4A21-54, which should render the processing at this site less NS4A-dependent. Still no cleavage was detectable at the cis cleavage site, NS3-NS4A, after addition of Pep4A21-54. However, processing was now also detectable at the NS4B-NS5A site, although at higher enzyme concentrations as those required for efficient cleavage of the other two trans sites.

Next, the cleavage of in vitro translated NS5A-NS5BΔC51 was used as an assay for testing the inhibitory potential of a series of protease inhibitors on NS3 (Fig. 4). Classical serine protease inhibitors such as PMSF and disopropyl fluorophosphate, the chymotrypsin inhibitors TPCK as well as apronin were effective in inhibiting the NS3 protease. On the other hand, other trypsin, chymotrypsin, cysteine- and metalloproteinase inhibitors were unable to yield significant inhibition of the activity of NS3. Together with previous mutagenesis studies (11), these findings about its reactivity confirm the identity of the NS3 protease as serine protease.

Activity on Peptide Substrates—Our next interest was to find out if the purified protease was able to cleave a synthetic peptide substrate. We excluded the NS3-NS4A site (as a cis cleavage site) and the NS4B-NS5A site (as an absolutely NS4A-dependent site) as possible candidates. Although being the most efficiently cleaved site we excluded also the NS5A-NS5B site due to its hydrophobicity and the problems expected from its two adjacent cysteine residues. As substrate we therefore tested a 20-mer peptide corresponding to the NS4A-NS4B junction (Pep4AB see "Materials and Methods"). The peptide was used as an Fmoc derivative, since this permitted fluorescence monitoring. Proteolytic cleavage of Pep4AB by the NS3 protease was detectable by HPLC, and occurred as expected after the P1 cysteine residue, since the cleavage products co-migrated with appropriate standards on HPLC. Furthermore, the isolated fragments, when analyzed by mass spectrometry, yielded the expected molecular masses (data not shown).

The cleavage efficiency was highly dependent on the detergent concentration in the assay mix, drastically declining at CHAPS concentrations below the critical micelle concentration value (not shown). The cleavage reaction of Pep4AB had a pH optimum around pH 8.5 and activity titration yielded an apparent pKα = 7.0, which are common values for most serine proteases.

We next addressed the question of whether synthetic NS4A analogs were able to increase cleavage efficiency of the purified protease also using Pep4AB as substrate. Furthermore, we wanted to verify that the minimum core region of NS4A is still capable of eliciting full activation of the isolated NS3 protease domain. To this purpose we compared the effects of Pep4A21-54 and the truncated peptides Pep4A33-54 and Pep4A21-34. As shown in Table I, Pep4A21-54 and Pep4A21-34, but not equivalent amounts of Pep4A33-54, were able to stimulate the activity, expressed as kcat/Km of the purified NS3 protease. These data directly confirm the region between amino acids 21 and 34 of NS4A as being responsible for the interaction with NS3 and show that the effect of NS4A is to enhance the efficiency of enzymatic catalysis. We wanted to further dissect the effect of NS4A by determining whether the peptide was increasing the affinity of the enzyme for its substrate or enhancing the catalytic rate. To this purpose substrate titration curves were fitted to the Michaelis-Menten equation and the kinetic parameters were calculated (Table I). These experiments demonstrate that Pep4A21-34 did not significantly affect Km values but acted on the rate of catalysis by increasing kcat values.

To evaluate the affinity of Pep4A21-34 for the NS3 protease a Pep4A21-34 titration experiment was done monitoring the rel-
activating rate enhancement (Fig. 5). Since we found the protease to be still monomeric after complex formation with NS4A (not shown), a 1:1 stoichiometry was assumed for the NS3-Pep4A21–34 complex. Based on this assumption an apparent $K_d$ of 0.22 $\mu M$ was calculated from a Scatchard plot (Fig. 5).

**DISCUSSION**

We here describe the purification of the hepatitis C virus NS3 protease domain from recombinant Baculovirus and the characterization of its enzymatic activity in vitro. The enzyme was found to be presumably membrane-associated in Sf9 cells, and detergent extraction was necessary to recover appreciable amounts of soluble protein.

The purified enzyme showed a very low specific activity, which is reminiscent of what has been reported for other viral proteases. As a matter of fact, both human cytomegalovirus protease ($k_{cat}/K_m = 5 \times 10^5 M^{-1} s^{-1}$, (27)) and herpes simplex virus protease ($k_{cat}/K_m = 17-37 M^{-1} s^{-1}$ (28, 29)) display cleavage kinetics that are comparable with the parameters we have determined for the HCV protease ($k_{cat}/K_m = 174 M^{-1} s^{-1}$).

While this manuscript was in preparation, two reports, describing the purification of fusion proteins encompassing both the protease and the helicase domains of the NS3 protein, were published (30, 31). One report (31) describes the activity on a peptide substrate corresponding to the NS5A-NS5B junction. The kinetic parameters that can be calculated from the published data ($k_{cat}/K_m = 100 M^{-1} s^{-1}$) are in good agreement with our own findings.

From transfection experiments the temporal hierarchy of cleavage events of NS3-dependent junctions within the non-structural region has been determined as being: NS3-NS4A, NS5A-NS5B, NS4A-NS4B, NS4B-NS5A. The latter cleavage being completely dependent on the presence of NS4A. It is likely that this hierarchy reflects physiological requirements of the viral life cycle that are still elusive. Nor do we understand what factors govern the different cleavage kinetics at the single sites. Many open questions probably will have to be addressed by means of kinetic studies on purified proteins. As a first approach in this direction we investigated the activity of the purified NS3 protease domain on precursor proteins bearing all cleavage sites and on a synthetic peptide substrate.

The purified enzyme showed the highest activity on in vitro translated NS5A-NS5B, followed by NS4A-NS4B, while NS4B-NS5A was not detectably cleaved. The latter precursor was cleaved only in the presence of a peptide corresponding to the 34 C-terminal amino acids of NS4A. The same peptide had only very modest effects on the cleavage efficiency at the NS5A-
FIG. 4. Effect of protease inhibitors on the processing of the NS5A-NS5B/C51 precursor by the purified protease. Pretranslated radiolabeled NS5A-NS5B/C51 precursor was incubated in the presence of purified NS3 protease and different canonical protease inhibitors as described under "Materials and Methods." Undeaved precursor and cleavage products were separated on an SDS-12% polyacrylamide gel and visualized by autoradiography. The percentage of cleavage in the presence of added inhibitors was determined by densitometric analysis and data were expressed as percent residual activity with respect to appropriate control samples. Inhibitors and their final concentrations were: DFP, diisopropyl fluorophosphate (1 mM or 10 mM); PMSF, phenylmethylsulfonyl fluoride (1 mM); TPCK, tosylphenylalanyl chloromethyl ketone (1 mM); TLCK, N\textsuperscript{a}-tosyl-L-lysine chloromethyl ketone (0.5 mM); aprotinin (0.5 mg/ml); chymostatin (0.5 mg/ml); phenanthrolone (2 mM); EDTA (2 mM); ZnCl\textsubscript{2} (2 mM); leupeptin (0.5 mg/ml). Data are from one experiment representative of three.

TABLE 1

| Addition            | $K_m/K_{cat}$ | $k_{cat}$ | $K_m$ |
|---------------------|---------------|-----------|-------|
| None                | 10.460 ± 127  | 0.32 ± 0.01 | 29.9 ± 1.3 |
| Pep4A\textsubscript{21–34} | 26,754 ± 802 | ND        | ND    |
| Pep4A\textsubscript{33–54} | 12,432 ± 755 | ND        | ND    |
| Pep4A\textsubscript{21–35} | 33,040 ± 1590 | 0.86 ± 0.03 | 30.3 ± 3.5 |

Effect of Pep4A-derived peptides on the cleavage kinetics of Pep4A

The major effect of NS4A to be on the catalytic rate constant $k_{cat}$.

This rate constant could be increased due to structural rearrangements altering the nucleophility of the active site serine residue or affecting transition state binding. It has to be pointed out, however, that these kinetic differences were observed using a peptide derived from an NS4A-independent cleavage site. Comparison of the very small effects of Pep4A\textsubscript{21–34} on the processing efficiency of the in vitro translated NS4A-NS4B precursor with the absolute Pep4A\textsubscript{21–34} requirement for NS4B-NS5A precursor processing (Fig. 3) suggests that different mechanisms might account for the effects of NS4A on the processing at the single cleavage sites.

The interaction domain with NS4A has been mapped to the N terminus of NS3 (21, 22). In this work we have addressed the question of what region of NS4A interacts with NS3. A recent report, using a recombinant vaccinia/transfection system comes to the conclusion that a 13-amino acid region spanning residues 22–34 of NS4A is crucial for the interaction with NS3 (23). These findings are confirmed by our observation that deletion of 12 amino acids at the N terminus of Pep4A\textsubscript{21–54} abolishes its ability to activate NS3, strongly arguing for an involvement of these residues in the interaction with NS3. A further proof of this assumption is given by the fact that the 14-mer peptide Pep4A\textsubscript{21–34} binds with high affinity to the purified protease and has a potential of activating NS3, which is undistinguishable from the effect observed upon addition of Pep4A\textsubscript{21–54}.

It is interesting to notice that structure predictions of NS4A predict two $\alpha$-helices with a highly hydrophobic region in their middle. This prediction has been partially confirmed by...
CD spectra of Pep4A21–54. 

Notably, the residues which are apparently crucial for the interaction with NS3 fall exactly in this region, indicating that a hydrophobic extended structure of NS4A contacts the N-terminal domain of NS3. Proteolytic events mediated by the NS3 protease are likely to be absolute requirements for the generation of an active viral replication apparatus. Sequence alignments point to NS5B as harboring this region, indicating that a hydrophobic extended structure of apparently crucial for the interaction with NS3 fall exactly in this protein is generated by an NS3-dependent cleavage. Thus, this enzyme appears as being an attractive candidate target for the development of anti-HCV therapeutics. A deeper understanding of the regulation and the substrate requirements of the protease will help to develop first generation inhibitors.

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