In vivo and in vitro trans-cleavage activity of hepatitis C virus serine proteinase expressed by recombinant baculoviruses

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By the use of recombinant baculoviruses, the trans-cleavage of hepatitis C virus (HCV) non-structural polyprotein was studied. The viral serine proteinase encoded by the NS3 gene was expressed efficiently in insect cells infected with a baculovirus recombined with HCV cDNA corresponding to amino acids 1046–1243 and the signal sequence of the rabies virus G protein. Coinfection studies showed the in vivo trans-cleavage activity of the expressed protein by the use of a recombinant producing NS5 as a substrate. We also found that the partially purified NS3 serine proteinase prepared from the recombinant-infected cells could cleave NS5A/5B substrate. Characterization of the proteinase obtained will provide basic knowledge on processing of the HCV polyprotein.

Introduction

Infection with hepatitis C virus (HCV) is the major cause of both post-transfusion and sporadic non-A non-B hepatitis (Alter et al., 1992; Choo et al., 1989; Kuo et al., 1989). HCV contains a positive-stranded RNA genome of approximately 9400 bases. The genome consists of a single, large open reading frame encoding a polyprotein of 3010–3033 amino acids (aa). Although little overall primary sequence homology exists between HCV and other known viruses, HCV appears to be most closely related to flaviviruses and pestiviruses on the basis of similarities of its genomic structure and organization (Choo et al., 1991; Kato et al., 1990; Okamoto et al., 1991, 1992; Takamizawa et al., 1991). The order of the HCV polyprotein is considered to be NH₂-core (C)–envelope (E)₁–E₂–non-structural (NS) ₂–NS3–NS4A–NS4B–NS5A–NS5B–COOH.

Recent investigations revealed that four cleavages (NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B) on the HCV precursor polyprotein are mediated by a viral proteinase lying within the amino-terminal half of the NS3 protein, which is a serine-type proteinase of the trypsin superfamily (Bartenschlager et al., 1993; Eckart et al., 1993; Grakoui et al., 1993; Hijikata et al., 1993; Tomei et al., 1993). It has also been reported that three of these four cleavages can be achieved by supplying NS3 in trans, whereas the NS3/NS4A site is cleaved only in cis (Tomei et al., 1993). More recently, it was proposed that NS4A protein, in addition to NS3, is also required for cleavage at the NS3/4A and NS4B/5A sites and accelerates the rate of cleavage at the NS5A/5B site (Bartenschlager et al., 1994; Failla et al., 1994). A molecular model of substrate binding pocket of the NS3 serine proteinase was built on the basis of the comparative analysis of the structures of other known serine proteinases and the NS3 of different HCV strains. The ideal residues at the P₁ position of the cleavage site were proposed from the configuration of the pocket (Pizzi et al., 1994). The P₁ position of the polyprotein substrate was suggested to be more important in defining the specificity than the P₆ or P₁ position whose amino acid sequences are well-conserved among different HCV strains reported (Leinbach et al., 1994).

The proteinase activity of NS3 has been elucidated by in vitro transcription/translation systems or expression systems in cultured cells and the proteolytic processing of the HCV polyprotein was clearly shown. Although a lot of information has been obtained through these approaches, such studies are of limited value for detailed biochemical analysis of the proteinase. Isolation of NS3 serine proteinase is prerequisite to the study of properties such as substrate specificity and interaction with effectors.
including NS4A for the proteolytic activity. Experiments with isolated material would provide the fundamental understanding on processing of the HCV polyprotein.

We report here efficient expression of NS3 serine protease in a recombinant baculovirus system. The expressed protein exhibited trans-cleavage activity when it was coexpressed with NS5A/5B protein in insect cells. We also describe the specific proteolytic activity of partially purified NS3 protease.

**Methods**

**Cells and viruses.** *Trichophasia ni* (Tn) S cells were cultured in SF900II medium (Gibco BRL) at 26.5 °C. *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) and various recombinant baculoviruses were prepared and grown as described previously (Matsuura *et al.*, 1989). As a control virus, we constructed a deletion virus, AcdPol, from which both the promoter and coding region of the polyhedrin gene were removed. Transfer vector pAcYM1 (Matsuura *et al.*, 1987) was digested with EcoRV and *Bam*HI, filled with the Klenow fragment of *Escherichia coli* DNA polymerase I and self-ligated. The resulting pAcdPol was cotransfected with infectious AcMNPV DNA, then the polyhedrin-negative virus was isolated.

**Construction of plasmids for homologous recombination.** The HCV cDNAs used for expression were isolated by the nested polymerase chain reaction (PCR) (Takeuchi *et al.*, 1990) from the serum of a healthy HCV carrier in Japan. To generate pAc3440, construct pUC3241, in which HCV cDNA spanning nucleotides (nt) 3249 to 4147 (numbering system according to Kato *et al.*, 1990) was cloned into Smal-linearized pUC19, digested with *SspI* and *NdeI*, and the cDNA fragment (nt 3463-4058) was incubated with the Klenow fragment of DNA polymerase I, then ligated with a 12-mer *NcoI* linker (5' CAGCCATGCGCTG 3') to construct a translation initiation codon. The resulting fragment digested with *NcoI* was inserted into pAcYM10 which was ligated with the same *NcoI* linker at the Smal site of the pAcYM1 transfer vector (Matsuura *et al.*, 1987). To construct pAc6690, EcoRI-KpnI fragment (nt 6688 to 9069) was obtained from pBR394 in which full-length HCV cDNA was cloned into the HindIII site of pBR322. It was then blunt-ended and ligated with the 12-mer *NcoI* linker before insertion into the *NcoI* site of the pAcYM10.

Fig. 1. (a) Constructs of recombinant baculoviruses used in this study. The organization of HCV polyprotein is indicated at the top. Constructs expressing regions of HCV polyprotein are shown below. The signal peptide of rabies virus G protein is indicated by closed boxes. The numbers above the boxes indicate the positions of the N- or C-terminal amino acids of HCV polyprotein portions encompassed by recombinants. The names of recombinant baculoviruses are shown on the left. The asterisk in the construct AcRV3440/H1083A represents an amino acid substitution that changed the His residue at position 1083 to an Ala residue. (b) Nucleotide and amino acid sequences spanning the signal peptide of rabies virus G protein. These 72-mer oligonucleotides were synthesized and annealed, then the resulting double-stranded DNA fragment was inserted into the *Bam*HI site of pAcYM1.
To construct the transfer vectors encoding HCV proteins fused to the signal peptide of rabies virus G protein, we first modified the parental pAcYM1 by ligating a 72 bp double-stranded DNA fragment coding for the signal peptide (Fig. 1 b) to the BamHI site of the vector. In this modified transfer vector, which we named pAcYMRV, only one BamHI site remains downstream of the signal sequence. Construct pAcRV3440 contains the SpeI–NdeI fragment of HCV cDNA derived from pUC3241, which is the same as in pAc3440, cloned into the BamHI site of pAcYMRV by treatment with Klenow fragment before ligation.

A single amino acid mutation was introduced into the catalytic site of the NS3 serine protease. The mutation of His (aa 1083) to Ala was introduced using PCR with a sense primer (nt 3249–3268) and a mismatched antisense primer, 5' CGGCACCGGCAAAGACAGT-3' and pUC3241 as a template. The 120 bp SpeI–BamHI fragment of the amplified mutated DNA was recloned into pUC3241 using the same restriction sites. Construct pAcRV3440/H1083A is identical to pAc3440 except for the substitution mutation, His at aa 1083 to Ala. The presence of this mutation in the construct was verified by sequence analysis.

**Immunoblot analysis.** Infected cells were harvested at 48 h post-infection (p.i.) and lysed in Laemmli sample buffer (Laemmli, 1970), followed by SDS-PAGE. After transfer to a PVDF membrane (Millipore), the blots were blocked with non-fat dried milk and incubated with rabbit antisera or monoclonal antibodies diluted 1:100 to 1:1000. They were then incubated with biotinylated anti-rabbit or anti-mouse IgG and peroxidase-conjugated streptavidin and developed with 4-chloro-1-naphthol.

**Radiolabelling and immunoprecipitation.** Cells infected with the recombinant baculoviruses were cultured for 24 h and then starved by incubation in SF900II medium lacking methionine and cysteine for 2 h prior to the addition of 0.74 MBq of TRANZSS label (ICN). Cells were pulse-labelled for 15 min, washed and chased in TC100 medium for up to 3 h. Samples were harvested at various times and lysed with a solution containing 10 mM-Tris–HCl (pH 7.8), 150 mM-NaCl, 1 mM-EDTA, 1% NP40, 0.1% SDS and 10 mg/ml of aprotinin. HCV proteins in the cell lysates were immunoprecipitated with a monoclonal antibody (Manabe et al., 1994) against HCV NS5B protein, and the immunocomplexes were collected as described previously (Matsumura et al., 1994). The immunoprecipitates were separated by SDS–PAGE and the radioactivity was measured in a Bio-Image model BAS2000 analyser (Fuji Photo Film).

**N-terminal amino acid sequence analysis.** A cell extract coinfected with AcRV3440 and Ac6690 was separated on a 7.5% SDS–polyacrylamide gel. Proteins electrophoretically onto a PVDF membrane were stained with Coomassie blue and the expected band for processed NS5B protein was cut out. The membrane was placed in the cartridge block and the amino acid sequence was determined with an Applied Biosystems model 477A protein sequencer (Matsudaira, 1987).

**In vitro proteolytic assay with NS3 serine protease.** Approximately 2 x 10⁶ cells were infected with recombinant virus AcRV3440 or AcRV3440/H1083A, which encodes NS3 serine protease (aa 1046–1243) or its variant mutated in the catalytic site, respectively, at an m.o.i. of 5 p.f.u./cell. After 48 h, the infected cells were collected, washed with Tris-buffered saline (TBS) three times, and centrifuged at 1000 g for 10 min. The cell pellet was resuspended in 1 ml of 50 mM-Tris–HCl (pH 7.5), 5 mM-EDTA and 5 mM-EGTA (TE buffer) and homogenized by 15 to 20 strokes of a tight-fitting Dounce homogenizer. After centrifugation at 10000 g for 15 min, the supernatant was clarified by centrifugation at 10000 g for 15 min and the supernatant was examined for protease activity.

To prepare cell extracts as substrates, Tn5 cells (approximately 2 x 10⁷) infected with Ac6690 coding for a polyprotein extending from N-terminal truncated NS5A to C-terminal truncation of NS5B (aa 2121–2912) at an m.o.i. of 5 were cultured for 48 h. Cells were harvested by centrifugation, washed with TBS three times and lysed in 1 ml of 1% Tween-80/TE buffer with the Dounce homogenizer. After centrifugation at 10000 g for 15 min, the pellet was washed with TE buffer once and sonicated with a Branson Sonifier in 1 ml of 1% NLS/TE buffer. This lysate was centrifuged for 15 min at 10000 g and the supernatant was used as a substrate to detect enzymatic activity.

For standard proteolytic assay, partially purified NS3 proteinase or its mutated protein (approximately 1 μg of protein) was incubated with an extract from Ac6690-infected cells (approximately 2 μg of protein) at 30°C for 1–6 h. The reaction was carried out in 10 μl of 0.5% NLS/TE buffer and stopped by adding an equal amount of sample buffer for SDS–PAGE. Proteins were separated on a 7.5% SDS–polyacrylamide gel and subjected to immunoblot analysis using a monoclonal antibody directed against NS5B.

**Results**

**Efficient expression of NS3 serine proteinase in insect cells**

To express the HCV serine proteinase domain of NS3 efficiently, we attempted to express the HCV cDNAs with an N-terminal signal peptide coding sequence fused in-frame by using a baculovirus vector. The signal peptide sequence of rabies virus G protein was chosen because this protein is produced abundantly using the recombinant baculovirus system (Prehaud et al., 1989) and the signal is known to be cleaved in insect cells (Nishihara et al., 1993). Two transfer vectors bearing the N-terminal region of NS3 (NS3ΔC), aa 1046–1243, which is predicted to drive the expression of a polypeptide spanning the active proteinase domain, one (pAcRV3440) with the signal peptide and the other (pAc3440) without it, were constructed and inserted into AcMNPV by homologous recombination. These constructions and synthetic nucleotide sequences for the signal peptide are illustrated in Fig. 1(a and b), respectively. To examine the expression of NS3 serine proteinase, Tn5 cells were infected with either AcRV3440, Ac3440 or control virus (AcdPol) and cultured for 48 h. The infected cells were analysed by SDS–PAGE and stained with Coomassie blue (Fig. 2a). A predominant band at 29 kDa was detected in the cell extract from AcRV3440-infected cells but not in the extract from cells infected with AcdPol. Although Ac3440 also induced significant expression of this protein, its level was less than one-fourth of that with AcRV3440 as determined by densitometric scanning.

To ensure that the 29 kDa band was indeed HCV NS3 protein, immunoblot analysis was performed with a rabbit polyclonal antisera, anti-NS2/3, raised against...
Fig. 2. Expression of HCV NS3 serine proteinase in insect cells infected with recombinant baculoviruses. Tn5 cells were infected with recombinant baculoviruses Ac3440 (lane 1), AcRV3440 (lane 2) or AcdPol (lane 3) and the infected cells or culture supernatants were harvested at 48 h p.i. (a) The total cell extracts were separated by 15% SDS-PAGE and stained with Coomassie blue. (b) The total cell extracts (left) or culture supernatants (right) were separated by 15% SDS-PAGE and analysed by immunoblotting using a polyclonal antibody (anti-NS2/3). Arrows indicate NS3AC. The numbers at the left represent molecular weight markers.

a polypeptide spanning aa 1008–1243 (C terminus of NS2 to the N-terminal third of NS3). As shown in Fig. 2(b), the 29 kDa protein was clearly detected in cells infected with either Ac3440 or AcRV3440. The protein produced by AcRV3440 was much more abundant than that produced by Ac3440.

The molecular mass of NS3AC expressed in AcRV3440-infected cells was the same as in cells infected with Ac3440, which strongly suggested that the signal peptide inserted into AcRV3440 was cleaved in Tn5 cells. To determine whether this processed NS3AC is secreted into the culture medium, we analysed the culture supernatant of AcRV3440- or Ac3440-infected cells by immunoblotting. The immunoreactive 29 kDa protein was observed in the culture supernatant of AcRV3440-infected cells but there was only a little in that of Ac3440-infected cells (Fig. 2b).

As shown above, the present system using the signal peptide of rabies virus G protein is capable not only of efficiently expressing the amino-terminal third of NS3 but also of enhancing the secretion into the culture medium. However, more than 80% of the expressed protein was nevertheless located in the cells. Consequently, we used the NS3AC protein intracellularly expressed by AcRV3440 for the following studies to determine whether there is proteinase activity in vivo and in vitro.

Trans-cleavage at NS5A/NS5B site by NS3 serine proteinase in insect cells

To examine the trans-cleavage activity of NS3 under the conditions described above, we constructed recombinant baculovirus Ac6690 expressing a polyprotein truncated at the N terminus of NS5A (NS5AΔN) and the C terminus of NS5B (NS5BΔC) as a substrate. We also constructed AcRV3440/H1083A by introducing a single amino acid substitution at His-1083 to Ala to inactivate the serine proteinase of NS3 as well as AcRV3440. Tn5 cells were infected with either AcRV3440 or AcRV3440/H1083A together with Ac6690. The cleavage activities were assessed after 48 h incubation by immunoblotting in which monoclonal antibodies against NS5A or NS5B were used as probes (Fig. 3a and b). As expected, substrate cleavage products corresponding to the truncated forms NS5AΔN and NS5BΔC were identified in cells coinfected with AcRV3440 and Ac6690 (lanes 2). In contrast, no cleavage product or accumulation of the precursor protein were observed in the cells singly infected with Ac6690 (lanes 1) or coinfected with AcRV3440/H1083A and Ac6690 (lanes 3). This is not because of the low expression level of inactive NS3AC protein. The expression by AcRV3440/H1083A was as high as that of the active protein by AcRV3440 (Fig. 3 c).

In addition, the N-terminal sequence of the processed NS5BΔC protein was determined. Cells coinfected with AcRV3440 and Ac6690 were separated on a 7.5% SDS-polyacrylamide gel and transferred to a PVDF membrane, then the Coomassie blue-stained band corresponding to the processed NS5BΔC protein was subjected to protein sequencing. The N-terminal sequence obtained was in good agreement with those previously reported (Grakoui et al., 1993; Pizzi et al., 1994) i.e., the N terminus of the processed NS5BΔC protein was mapped at residue 2421 (Fig. 3 b, in parentheses). These results clearly show that the NS3ΔC expressed by AcRV3440 has trans-cleavage activity at the NS5A/NS5B site.
Cleavage activity of HCV serine proteinase

Fig. 3. In vivo processing of NS5 protein by NS3 serine proteinase. Tn5 cells were infected with Ac6690 expressing NS5AAN/NS5BAC (lane 1) or a combination of Ac6690 and AcRV3440 (lane 2) or AcRV3440/H1083A (lane 3) expressing enzymatically active or inactive proteinase, respectively. Expression and processing of HCV proteins were analysed by immunoblotting using monoclonal antibodies against NS5A (a) or NS5B (b). The N-terminal amino acid sequence of processed NS5B is shown in parentheses (b). (c) Expression of enzymatically active or inactive HCV NS3 proteins was examined by immunoblotting using a polyclonal antibody against NS2/3.

Fig. 4. Pulse-chase analysis of NS5 processing by NS3 serine proteinase. Cells were infected with a combination of Ac6690 (Ac6690) and AcRV3440 (RV3440) or AcRV3440/H1083A (H1083A). After 26 h of infection, proteins were metabolically radiolabelled for 15 min, then incubated in non-radioactive medium for 1, 2 or 3 h. Proteins were isolated from the lysate by immunoprecipitation with a monoclonal antibody against NS5B.

Fig. 4 shows the results of a pulse-chase experiment to determine the kinetics of NS5A/NS5B processing in insect cells. Coinfected Tn5 cells were labelled metabolically with TRANSlabel at 26 h p.i. At 15 min after the beginning of labelling, a discrete band of a 100 kDa precursor protein and a small amount of processed NS5BΔC were detectable in cells coinfecte...
Fig. 5. NS3 serine proteinase-mediated trans-cleavage can occur in vitro. (a) Coomassie blue staining of wild-type or mutated NS3AC prepared from cells infected with AcRV3440 (RV3440) or AcRV3440/H1083A (H1083A), respectively. After 48 h of the infection, cells were harvested and washed with TEE buffer and 1% Tween-80/TEE buffer. After centrifugation the pellet was suspended in 0.5% NLS/TEE buffer using a Dounce homogenizer, then the soluble fraction was analysed by 15% SDS-PAGE and used as an enzymatically active or inactive NS3 serine proteinase for the in vitro assay. The position of the NS3AC is indicated by the arrowhead, and the positions of molecular weight markers are indicated on the left. (b) In vitro proteolytic assay with NS3 serine proteinase of recombinant baculovirus-infected cell lysates. The NLS/TEE-soluble fraction from Ac6690-infected cells (about 2 µg) was incubated alone, with about 1 µg of wild-type NS3AC (RV3440), or with about 1 µg of mutated NS3AC (H1083A). The lysates were incubated at 30°C for up to 6 h, then the specific processing was determined by immunoblot analysis using a monoclonal antibody against NS5B. Lane C is the lysate from cells coinfected with Ac6690 and AcRV3440. Specific cleavage by wild-type NS3AC, namely, NS3 serine proteinase, is indicated by the diminution of NS5AAN/NS5BAC and the accumulation of NS5BAC.

The extract from either AcRV3440- or AcRV3440/H1083A-infected cells was incubated with substrate in vitro at 30°C for up to 6 h and tested for the proteolytic activity of NS3 serine proteinase. The extent of cleavage of the NS5 precursor protein was monitored by immunoblot analysis to detect the decrease of NS5AAN/NS5BAC and the accumulation of processed NS5BAC (Fig. 5b). The extract derived from AcRV3440-infected cells was capable of cleaving the substrate in accordance with incubation period. We confirmed the identity of the processed product by immunoreaction with anti-NS5B monoclonal antibody and comigration with the NS5BAC processed in cells coinfected with AcRV3440 and Ac6690 as described above (lane C). In contrast, incubation of the extract from Ac6690-infected cells alone or with the extract from AcRV3440/H1083A-infected cells did not show any detectable proteolytic activity. These results proved that NS3 serine proteinase prepared under our conditions is active in vitro and is capable of catalysing trans-cleavage at the NS5A/NS5B site.

The trans-cleavage activity of NS3 serine proteinase over a pH range (5.5–9.0) was examined. The proteolytic activity was observed from pH 6.5 to 9.0 and was highest at about pH 8.0 (Fig. 6). Its activity was mostly lost below pH 6.0. These data show that NS3 serine proteinase has an optimum pH in the neutral to slightly basic region, but is active over a relatively broad range.

Discussion

The HCV NS3 serine proteinase, similar to other viral or cellular serine proteinases, is characterized by the presence of a catalytic triad composed of His, Asp and Ser which acts as the nucleophile during the cleavage of the scissile peptide bond. All the HCV strains heretofore
Molecularly cloned have this structure in common in their NS3 region (Grakoui et al., 1993; Hijikata et al., 1993; Miller & Purcell, 1990; Pizzi et al., 1994). Many studies of cell-free transcription/translation of HCV cDNAs and their expression in cultured cells support this (Bartenschlager et al., 1993; Eckart et al., 1993; Grakoui et al., 1993; Hijikata et al., 1993; Tomei et al., 1993). It has been demonstrated that there are at least four cleavage events in HCV polyprotein processing by viral serine protease residing in the N-terminal third of NS3. More recently, it was found that the C terminus of NS3 (NS3/NS4A) is intramolecularly cleaved, whereas the other sites (NS4A/NS4B, NS4B/NS5A and NS5A/NS5B) are cleaved in trans by the serine protease (Tomei et al., 1993). The domain required for its trans-cleavage activity was also identified as the 167 residues from aa 1049–1215, by using a transient expression system in cultured cells (Tanji et al., 1994).

We have established an efficient system for expression of HCV serine protease using a recombinant baculovirus possessing the HCV cDNA encoding aa 1046–1243 fused to the sequence encoding the signal peptide of rabies virus G protein. In an earlier study, under the control of AcMNPV polyhedrin promoter this rabies G protein was expressed to a level accounting for about 18% of the total protein of the insect cell (Prehaud et al., 1989). Recently, E2 protein of HCV expressed by recombinant baculoviruses with this signal sequence was found to be secreted into the culture medium (Nishihara et al., 1993). In our system, the use of the signal peptide of rabies virus G protein increased the production of HCV NS3 serine protease and its secretion into the culture supernatant of insect cells. A high level expression of the protease was obtained representing at least 20% of the total cellular protein, by infection with AcRV3440. Extracellular secretion of the expressed protease was also enhanced. However, its level was relatively low; 2–10 μg/ml of culture medium judged by Coomassie blue staining.

To test the protease activity of the expressed protein, we performed a trans-cleavage assay by use of Ac6690 expressing 70% of the NS5 protein (aa 2121–2912) as a substrate. As expected, coinfection by Ac6690 and AcRV3440 yielded proteins corresponding to processed NS5AAN and NS5BAC. In contrast, no processed products were observed in coinfection by Ac6690 and AcRV3440/H1083A expressing an inactive NS3 serine protease. The result of N-terminal sequencing of the processed NS5BAC was consistent with previous studies using a vaccinia virus expression system (Grakoui et al., 1993; Pizzi et al., 1994). The kinetic study with the pulse-chase experiment revealed rather efficient processing, most of the precursor NS5AAN/NS5BAC was processed during the chase period of 3 h. This pattern is similar to one recently obtained by using recombinant vaccinia viruses expressing NS3 (aa 1007–1647) and NS3-NS5 (aa 1007–3011, including a substitution mutation in the serine protease domain) as a protease and a substrate, respectively (Bartenschlager et al., 1994). These data indicate that 198 aa of the N-terminal region of NS3 (aa 1046–1243) efficiently expressed by a recombinant baculovirus are sufficient for trans-cleavage at the NS5A/5B site.

The same expressed protein was capable of cleaving the NS5A/5B substrate in vitro. In our baculovirus expression system, more than 60% of the NS3AC expressed by AcRV3440 was detected in the intracellular insoluble fraction when it was prepared in the absence of detergents. A similar observation for the expression of whole NS3 protein was reported previously (Hirowatari et al., 1993). We tested several detergents to solubilize NS3AC and found that an enzymatically active protein could be prepared when the lysate was solubilized with 0.5% NLS. Moderate activity was also recovered from cell lysates with 1% Triton X-100, 1% n-octylglucoside, 1% CHAPS or 1% CHAPSO; lysates prepared with 1% NP40 or 1% Tween-80 were only slightly active, like that without any detergent (unpublished observation).

In this way, we could obtain the active HCV NS3 serine protease, which was more than 50% of the total protein in the extract, when the Tween-80-insoluble fraction from AcRV3440-infected cells was solubilized with 0.5% NLS. The substrate NS5AAN/NS5BAC was efficiently cleaved in the presence of NS3AC mentioned above, whereas the mutant NS3AC, in which His-1083 was changed to Ala, failed to cleave the substrate. This confirmed that the NS3 serine protease itself, prepared under our conditions, is responsible for the observed processing.

The NS3 serine protease has characteristics both similar to and different from the trypsin family as follows: it was proved through mutational analyses that a reactive Ser residue and two additional amino acids (Asp and His) are essential for cleavage at NS3/4A, NS4A/4B, NS4B/NS5A and NS5A/NS5B (Bartenschlager et al., 1993; Eckart et al., 1993; Grakoui et al., 1993; Hijikata et al., 1993; Tomei et al., 1993); the optimum pH of NS3 protease is neutral to slightly basic as presented here, which is similar to that of well-characterized serine proteases such as trypsin, chymotrypsin or thrombin; as for the substrate specificity, it was proposed that amino acid requirements for the P1 position are restrictive and that Cys and Ser residues are ideal at that position for the substrate (Grakoui et al., 1993; Leinbach et al., 1994; Pizzi et al., 1994); cellular serine proteases, however, cleave mainly after basic residues and these amino acids are usually found at the P1 and P2 positions for the substrates of flaviviral serine

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partially purified HCV NS3 serine proteinase, is currently being used in further biochemical and structure determination studies. The basic information generated, especially on the features which distinguish HCV NS3 from other serine proteinases, will give us ways to search for selective inhibitors of the HCV serine proteinase.

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