Hepatitis C virus NS3 serine protease interacts with the serpin C1 inhibitor

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Abstract Both NS3 protein (1007–1657) and its protease moiety (NS3p, 1027–1207) were able to interact in vitro with C1 Inhibitor (C1Inh) to give a 95-kDa M_r C1Inh cleavage product similar to that obtained upon proteolysis by complement protease C1s. High-M_r reaction products were also detected after incubation of C1Inh with NS3 but not with NS3p; they correspond to ester-bonded complexes from their hydroxylamine lability. Similar reactivity of NS3 was observed upon incubation with α2-antiplasmin. Serpin cleavage was prevented by treatment of NS3 with synthetic serine protease inhibitors. This interaction between viral NS3 and host serpins suggests that NS3 is likely to be controlled by infected cell protease inhibitors.

Key words: Hepatitis C virus; Serine protease; Non-structural protein 3; Serpin

1. Introduction

Hepatitis C virus (HCV) is the major aetiologic agent of non-A, non-B viral hepatitis [1,2]. The virus contains a single-stranded, positive-sense RNA genome of approximately 9600 nucleotides length. The single polyprotein precursor of 3010–3033 amino-acid residues is co- and post-translationally processed by cellular and viral proteases to yield the mature structural and non-structural proteins [2,3]. The structural proteins comprise the core protein and two envelope glycoproteins, E1 and E2, which are processed by the signal peptidase of the endoplasmic reticulum. Cleavage at the NS2/3 site is mediated by a protease composed of NS2 and the amino-terminal one-third of NS3. The serine protease (Ser-protease) NS3 is responsible for downstream cleavages in the non-structural region [4,5]. This protease activity is thought to be necessary for the viral replication and appears as a valid target for antiviral agents.

The N-terminal 180 amino acid region of NS3 has sequence homologies with the active sites of Ser-proteases [6–8]. His-1083, Asp-1107 and Ser-1165 (numbering according to their locations in the polyprotein of HCV subtype J/HCV 1b [9]) are considered as a catalytic triad similar to other Ser-proteases of the chymotrypsin family.

Ser-protease inhibitors or serpins are a large family of proteins which have been identified from various sources such as viruses and mammals [10–12]. They have been known for a long time as inhibitors of extracellular Ser-proteases; recent evidences suggest that a large number of serpins have intracellular activities: Squamous cell carcinoma antigen 2 [13] or cytotoxic cell proteinase inhibitor (PI)-6 [14] regulates the lysosome/granule protease cathepsin G, PI-9 inhibits granule granzyme B [15]. Moreover the myxoma virus serp2 or the cowpox cytokine response modifier A are able to regulate the caspase activity involved in apoptosis [16,17], indicating that viral serpins are able to regulate target cell proteases.

The serpin inhibitory mechanism involves the formation of an initial non-covalent complex followed by intermediate substrate-like cleavage of the serpin reactive centre loop and a tight serpin-protease covalent complex [11,12,18]. This complex is formed when the C-terminal P1 residue of cleaved serpin develops ester links with the active site Ser of the protease [11,12].

A control of pathogen protease by host cell serpin in the virus-host cell relationship has not been reported as yet. It becomes of interest to analyse a possible interplay between serpins and the HCV Ser-protease NS3. We give evidence that NS3 can interact with serpins such as C1 Inhibitor (C1Inh) and α-2 antiplasmin (α2AP), with proteolysis of the serpins and production of high-M_r complexes. This observation opens the way to pharmacological control on a new basis in an attempt to block HCV proliferation.

2. Materials and methods

2.1. Construction of expression plasmids

NS3 and its protease N terminal moiety (NS3p) were expressed as fusion proteins containing six His residues attached to the C terminus for Ni-chelating affinity purification. PCR-products were generated using pTM1007 (isolate G01, type 1b) as a template [19] and the oligonucleotide pairs: pair 1 (5′oligo: dCCCAAACAGTCGACTTCAG, 3′oligo: dTTTAGATCTCGTGACGACCTCCAGG) introducing a *Sal*I site at the 5′-end and a *Bgl*II site at the 3′-end of the sequence for the NS3 protein and pair 2 (5′ oligo: dGGCGACCCATGGCGCATATTACGGCC; 3′oligo: dGAAGACAGATCTCCGCATAGTGGTTTCC) introducing a *Nco*I site at the 5′-end and a *Bgl*II site at the 3′-end of the sequence for NS3p. The PCR products were ligated into the pQE_{60} vector (Qiagen, Paris, France) using the *Nco*I and the *Bgl*II restriction sites, and for NS3 protein with the addition of the *Nco*I-*Sal*I 1300-bp fragment prepared from pTM1007. The resulting plasmids encode the 1007–1657 sequence in the polyprotein, corresponding to the whole NS3 protein with a 20-residue N-terminus belonging to NS2 or the Ser-protease moiety of NS3 (1027–1207).

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Abbreviations: α2AP, α2 antiplasmin; C1Inh, C1 Inhibitor; DFP, diisopropylfluorophosphate; HCV, hepatitis C virus; NS3p, protease moiety of NS3; PMSF, phenylmethylsulfonyl fluoride; Ser-protease, serine protease; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, N^{α}-tosyl-Lys chloromethyl ketone; TPCK, N^{α}-tosyl-Phe chloromethyl ketone

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2.2. Expression and purification of recombinant NS3 and polypeptide fragments

Expression was induced and proteins were purified as described in [20]. Briefly bacteria were centrifuged, the pellet was freezed at −80°C, resuspended in 12 ml sonication buffer (300 mM NaCl, 1 mM 2-mercaptoethanol, 10 mM Na_2HPO_4, pH 7.4) and subjected to sonication (6 μm amplitude, energy 60, 3 cycles of 30 s each, Vibracell Bioblock, Illkirch, France) on ice. The sonicate was separated on a gel filtration column (Sephacryl-S300, Amersham Pharmacia Biotech, Saclay, France) and each NS3 protein was purified on the Ni-NTA affinity matrix (Qiagen, Paris, France) using a 0–150 mM imidazole gradient in the same buffer. Their purity was assessed from SDS-PAGE analysis and microsequencing. Due to imidazole interference, the protein in the samples was evaluated with ovalbumin as a standard on polyacrylamide gels. A single band was observed in purified samples with $M_r \sim 75$ kDa and ~ 25 kDa, respectively for NS3 and NS3p (Fig. 1). The proteolytic activity was assessed on recombinant NS5 protein (gift from Dr D. Vallari, Abbott, North Chicago, USA) as described by Steinkühler et al. [21] for a NS4A/B peptide.

2.3. Interaction NS3-serpins: incubation and analysis

C1Inh was purified according to Sim and Reboul [22], α2AP was purchased from Stago (Asnières, France). The serpins were incubated for 18 h at 37°C with variable amounts of NS3 or NS3p, in 15 μl 80 mM NaCl, 0.2 mM 2-mercaptoethanol, 0.1 mM $ZnCl_2$, 10 mM imidazole, 10 mM Na_2HPO_4, 10 mM Tris-HCl, pH 7.0. When indicated, NS3 was pre-treated with different concentrations of Ser-protease inhibitors (N^{α}-tosyl-Phe chloromethyl ketone (TPCK) or N^{α}-tosyl-Lys chloromethyl ketone (TLCK), Calbiochem, Meudon, France; di-isopropylfluorophosphate (DFP) or phenylmethylsulfonyl fluoride (PMSF), Sigma, Chesnes, France). In the case of deglycosylated C1Inh, the serpin was pre-incubated with *N*-glycosidase F (Roche-Boehringer, Meylan, France) in a glycosidase/C1Inh ratio of 3:100 (w/w) for 3.5 h at 37°C in 1 mM EDTA, pH 8.0.

After incubation of NS3 with serpins, samples were subjected to SDS-PAGE according to Laemmli [23]. For immunoblotting, the proteins were transferred to Immobilon membranes (Millipore, St Quentin-Yvelines, France) and subsequently incubated in 150 mM NaCl, 0.1% (w/v) Tween 20, 10 mM Tris-HCl, pH 7.5 with the peroxidase-labelled anti-serpin antibody under stirring for 2 h at room temperature. The membrane was washed (4×5 min) in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 and bands revealed with the 4-chloro-1-naphthol (Sigma, Chesnes, France). Alternatively a FITC-labelled antibody was used and the membrane subjected to FluorImager densitometry (Molecular Dynamics, Bondoufle, France).

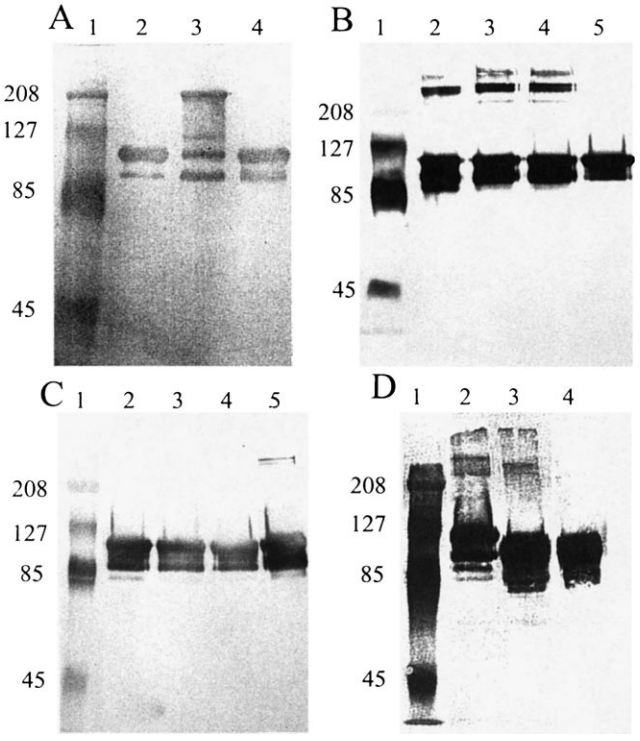

Fig. 2. C1Inh-NS3 interactions: analysis by SDS-PAGE and anti-C1Inh immunoblotting. The samples were loaded onto a SDS-7.5% polyacrylamide gel and subsequently transferred onto Immobilon membranes and incubated with a peroxidase-labelled anti-C1Inh antibody. A: Incubation assay of C1Inh (1 μg) with NS3p. Lanes: 2, C1Inh alone; 3, C1Inh+C1s (1.2 μg); 4, C1Inh+NS3p (3 μg). B: Assay of C1Inh (1 μg) with NS3. Lanes: 2, C1Inh/NS3 1:1; 3, C1Inh/NS3 1:2.5; 4, C1Inh/NS3 1:3.5; 5, C1Inh alone. C: Assay of C1Inh (1 μg) with ΔNS3. Lanes: 2, C1Inh+NS3p (3 μg); 3, C1Inh alone; 4, C1Inh+ΔNS3 (2 μg); 5, C1Inh+NS3 (1.2 μg). D: Assay of partially deglycosylated C1Inh (1 μg) with NS3. Lanes: 2, C1Inh+NS3 (1.2 μg); 3, deglycosylated C1Inh+NS3 (1.2 μg); 4, deglycosylated C1Inh. Lanes 1: M_r markers.

3. Results

3.1. Proteolytic cleavage of C1Inh and formation of high-M_r associations

To identify possible host serpin target for NS3, serpin-NS3 interaction was assessed by in vitro incubation of NS3 proteins with serpin in concentration comparable to that observed in extracellular fluids, followed by SDS-PAGE analysis and anti-serpin immunoblotting. 1 μg C1Inh was incubated with 3 μg NS3p (serpin/NS3p molar ratio of 1:1) or 1.2–4.2 μg NS3 (serpin/NS3 1:1-1:3.5) as described in Section 2. The samples were then subjected to SDS-PAGE and anti-C1Inh immunoblot analyses. Fig. 2A shows that C1Inh is susceptible to cleavage by NS3p with production of a band of $M_r \sim 95$-kDa band similar to that obtained after a 15-min incubation at 37°C with 1.2 μg C1s (C1Inh/C1s ratio of 1:1). The same band is observed after incubation with NS3, the maximum cleavage was obtained with serpin/NS3 molar ratio of 1:1 (Fig. 2B). In addition, high-M_r bands are clearly visible in the assay samples. The proteolysis product is also detectable after Coomassie blue staining (not shown). In the same conditions, C1Inh was incubated with a recombinant NS3 protein lacking the protease moiety (ΔNS3, sequence 1192–1458, a kind gift from Dr G. Baccala, Lyon, France) as negative control. Fig. 2C shows that ΔNS3 does not cleave C1Inh. These data indicate that C1Inh behaves as a substrate of NS3, with also formation of high-M_r associations between NS3 and the cleaved serpin.

The ester link between protease and serpin is labile to hydroxylamine at alkaline pH [24]. To analyse the high-M_r complexes, the samples, prepared as above at the serpin/NS3 mo-

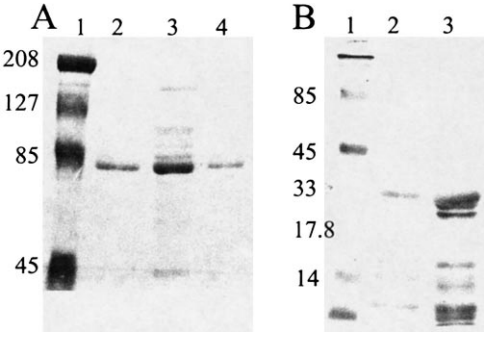

Fig. 1. Purification of the recombinant proteins. Samples were loaded on a SDS-10% polyacrylamide gel and proteins stained by Coomassie blue. Lanes: 1, M_r markers; 2, 3 and 4, fractions eluted at 20 mM imidazole. A: Purification of NS3 protein. B: Purification of NS3p.

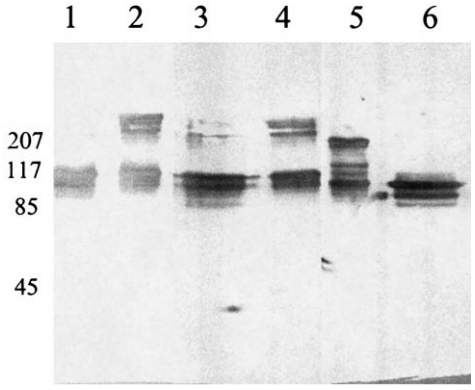

Fig. 3. Effect of hydroxylamine on high-M_r associations with C1Inh. The samples were loaded onto a SDS-7.5% polyacrylamide gel, transferred onto an Immobilon membrane, incubated with a FITC-labelled antibody and subjected to an FluorImager. Lanes: 1, C1Inh alone (1.5 μg); 2, C1Inh (1.5 μg)+NS3 (2 μg); 3, id_o and subjected to 1.2 M hydroxylamine pH 9.0; 4, id_o to 1.2 M NaCl pH 9.0; 5, C1Inh (1.5 μg)+C1s (2 μg) and subjected to 1.2 M NaCl pH 9.0; 6, id_o to 1.2 M hydroxylamine pH 9.0.

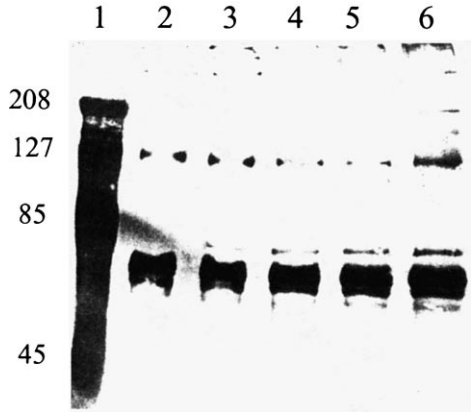

Fig. 5. α2AP-NS3 interaction: analysis by SDS-PAGE and immunoblotting. The samples were loaded onto a SDS-7.5% polyacrylamide gel and transferred onto an Immobilon membrane and incubated with a peroxidase-labelled anti-α2AP antibody. Lanes: 1, M_r markers; 2, α2AP alone (0.5 μg); 3, α2AP+NS3 with α2AP/NS3 ratio of 5:1; 4, α2AP/NS3 2:1; 5, α2AP/NS3 1:1; 6, α2AP/NS3 0.5:1.

lar ratio of 1:1, were subjected to 1.2 M hydroxylamine (pH 9.0) or to 1.2 M NaCl, 20 mM Tris-HCl, pH 9.0 (control) for 2 h at 37°C. Fig. 3 shows that high-M_r bands were decreased with subsequent increase of the 95-kDa band intensity (lane 3), quite similarly to the reference C1Inh-C1s complexes (lane 6). These data indicate that ester linkage was involved in high-M_r association between C1Inh and NS3.

3.2. Partially deglycosylated C1Inh is a target of NS3

Secreted C1Inh is highly glycosylated (26% carbohydrate w/w). Underglycosylated intracellular forms of C1Inh [25] are likely to coexist with NS3 in the cells infected by HCV. It was thus of interest to investigate if partially deglycosylated C1Inh could in vitro interact with NS3. C1Inh was treated by *N*-glycosidase F as described in Section 2 before incubation with NS3 (serpin/NS3 molar ratio of 1:1) as above. Fig. 2D shows that the underglycosylated C1Inh was cleaved in a ~80-kDa band with also formation of high M_r-bands upon incubation with NS3, as with C1s, indicating that the deglycosylated C1Inh is also able to interact with NS3.

3.3. Effects of synthetic protease inhibitors on the NS3-C1Inh interaction

To assess the proteolysis of C1Inh by NS3, the protease was preincubated in the presence of four synthetic Ser-protease inhibitors (TPCK, TLCK, DFP and PMSF) before addition of C1Inh: samples of 1.2 μg NS3 were treated with 0.05–0.2 mM TPCK, 0.2–2 mM TLCK, 5–10 mM DFP or 2 mM PMSF for 30 min at 37°C before incubation with 2 μg C1Inh in the same conditions as above. SDS-PAGE and anti-C1Inh immunoblot analyses of the samples showed that the proteolysis of C1Inh by NS3 was susceptible to 10 mM DFP, 0.2–2 mM TLCK (Fig. 4, lanes 2, 5 and 6) and 2 mM PMSF (not shown) and partially susceptible to 0.2 mM TPCK (Fig. 4, lane 8). These data confirm that the NS3 proteolysis of C1Inh is due to the Ser-protease NS3.

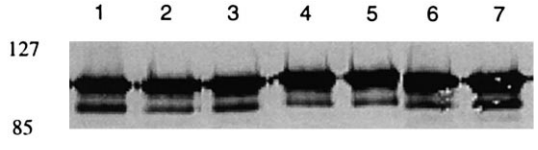

Fig. 4. Susceptibility of proteolysis of C1Inh by NS3 to Ser-protease inhibitors. Lanes: 1, C1Inh (1 μg)+NS3 (1.2 μg); 2, id_o after incubation of NS3 with 10 mM DFP; 3, 5 mM DFP; 4, 2 mM TLCK; 5, 0.2 mM TLCK; 6, 0.2 mM TPCK; 7, 0.05 mM TPCK.

3.4. Interactions of NS3 with α2 antiplasmin

To check that the reactivity of NS3 was not restricted to C1Inh, samples of α2AP, another serpin, were incubated with NS3 (serpin/NS3 molar ratios of 0.5:1 to 5:1) in the above conditions and subsequently analysed by SDS-PAGE and anti-α2AP immunoblot. Fig. 5 shows that α2AP was also susceptible to cleavage by NS3 with production of a band of $M_r \sim 65\,000$ and with also formation of high-M_r complexes.

The same procedure was applied to α1-anti-trypsin and ovalbumin (a non-inhibitory serpin) without evidence for their cleavage by NS3 (not shown). These data indicate that NS3 is able to interact with several but not all serpins.

4. Discussion

Reactive centres of serpins consist of a mobile loop with P1-P′1 residues behaving as targets for a number of proteases. NS3 interacts with two serpins, C1Inh and α2AP with production of fragment of similar size to those produced upon cleavage by other proteases. Formation of intermediate high-M_r complexes characteristic of the serpin activity, according to a structural model proposed recently [18], has also been reported. This suggests that the reactive centre loops of C1Inh and α2AP are two other possible substrates for NS3 beyond the already known cleavages of the HCV polyprotein [4,5,26]. This cleavage of C1Inh and α2AP is achieved by the Ser-protease site of NS3, from its inhibition pattern by specific synthetic inhibitors which is similar to the inhibition pattern of NS3-catalysed HCV polypeptide cleavage [21]. The observation of high-M_r association is the best evidence for a control of NS3 proteolytic activity by serpins. High-M_r associations were only detected after incubation with complete NS3

and not with NS3p, which probably reflects a role of the large C-terminal helicase moiety of NS3 in stabilising serpin-protease complexes. This could be related to a functional interdependence between the two protease and helicase moieties of NS3, as it has been suggested by the polynucleotide modulation of the protease activity [27].

These results with both C1Inh and α2AP represents the first in vitro observation between purified host serpins and the HCV NS3 protease. As the two serpins are synthesised and secreted mainly by hepatocytes, their role in hepatitis C can be questioned: is a deficiency of a serpin associated with increased risk for hepatitis C? Up to now, dealing with another serpin, α1-antichymotrypsin, a relationship between its deficiency and chronic HCV infection has not been described, although low plasma levels of α1-antichymotrypsin were frequently observed [28]. Our observation however supports the hypothesis that HCV NS3 produced by infected hepatocytes is likely to be regulated by host cell proteins such as serpins. This hypothesis is currently explored in an in vitro system of cultured hepatoma cells producing C1Inh in which HCV NS3 is expressed.

Control of HCV NS3 protease by host serpins can be viewed as a general mechanism where serpins of target cells act as sentinels of innate immunity against viral infections. It appears thus of interest to refine anti-HCV therapy by promoting or reinforcing interaction of the serpins described here, and eventually of other serpins, with the HCV NS3 protease inside hepatocytes or other targets of the virus.

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