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Antiviral Research 40 (1998) 1–18

Antiviral Research

Mini review

Hepatitis C virus NS3/4A protease

Ann D. Kwong [a,*], Joseph L. Kim [a], Govinda Rao [a], Dasa Lipovsek [b], Scott A. Raybuck [a]

[a] *Vertex Pharmaceuticals, Inc., 130 Waverly Street, Cambridge, MA 02139, USA*
[b] *Phylos, Inc., 300 Putnam, Cambridge, MA 02139, USA*

Received 13 June 1998; accepted 15 July 1998

Abstract

Despite an urgent medical need, a broadly effective anti-viral therapy for the treatment of infections with hepatitis C viruses (HCVs) has yet to be developed. One of the approaches to anti-HCV drug discovery is the design and development of specific small molecule drugs to inhibit the proteolytic processing of the HCV polyprotein. This proteolytic processing is catalyzed by a chymotrypsin-like serine protease which is located in the N-terminal region of non-structural protein 3 (NS3). This protease domain forms a tight, non-covalent complex with NS4A, a 54 amino acid activator of NS3 protease. The C-terminal two-thirds of the NS3 protein contain a helicase and a nucleic acid-stimulated nucleoside triphosphatase (NTPase) activities which are probably involved in viral replication. This review will focus on the structure and function of the serine protease activity of NS3/4A and the development of inhibitors of this activity.

Keywords: Hepatitis C; Treatment; Protease activity

1. Introduction

HCV is an enveloped, positive-stranded RNA virus with a linear RNA genome of approximately 9.6 kb (Kaito et al., 1994). The HCV genome encodes a single large polyprotein of approximately 3000 amino acids. Proteolytic processing by both host signal peptidases and viral proteases results in at least ten viral proteins in the order: NH_2–C–E1–E2–p7–NS2–NS3–NS4A–NS4B–NS5A–NS5B–COOH (Hijikata et al., 1991; Grakoui et al., 1993; Lin and Rice, 1995). Putative essential HCV replication proteins and genomic secondary structures such as the internal

* Corresponding author. Tel.: +1 617 5776319; fax: +1 617 5776210; e-mail: kwong@vpharm.com

0166-3542/98/$ - see front matter
PII S0166-3542(98)00043-6

ribosome entry site (IRES) and the 3′-UTR as targets for anti-HCV therapy have been recently reviewed (Bartenschlager, 1997; Clarke, 1997). One putative essential protein of HCV is NS3, a multifunctional enzyme. The N-terminal domain of NS3 contains a chymotrypsin-like serine protease which is required for proteolytic processing of four cleavage sites in the nonstructural region of the HCV polyprotein (Bartenschlager et al., 1993; Grakoui et al., 1993; Tomei et al., 1993). The C-terminal two-thirds of the NS3 protein contain both a helicase (Bartenschlager et al., 1993; Grakoui et al., 1993; Tomei et al., 1993; Kim et al., 1995; Hong et al., 1996) and a nucleic acid-stimulated nucleoside triphosphatase (NTPase) (Bartenschlager et al., 1993; Grakoui et al., 1993; Suzich et al., 1993; Tamura et al., 1993; Tomei et al., 1993; Kim et al., 1995; Hong et al., 1996; Preugschat et al., 1996; Morgenstern et al., 1997). NS3 forms a tight non-covalent complex with NS4A, a 54 amino acid activator of NS3 protease.

Due to the lack of an efficient and robust cell culture system for HCV, genetic knockout experiments which would prove that the NS3 gene of HCV encodes an essential protein have not yet been reported. However, proteolytic processing of non structural (NS) proteins in the polyprotein of genetically related viruses from the Flaviridae family is known to be performed by viral encoded proteases, which are essential for the infection and propagation of these viruses (reviewed in Hollinger (1990)). Studies with Yellow Fever virus (YF) have demonstrated that mutations which abolish the NS3 protease activity (Chambers et al., 1991) are lethal for YF virus replication.

Despite urgent medical need, a broadly effective anti-viral therapy for the treatment of infections with hepatitis C viruses (HCVs) has yet to be developed. In part because of the proven success of protease inhibitors to treat HIV disease, a large number of pharmaceutical and biotechnology companies have focused on the HCV NS3 protease as a target for small molecule anti-viral drug development. Heightened interest in structure-based drug design has been fueled by the publications of the structures of the NS3 serine protease domain in the absence (Love et al., 1996) and presence (Kim et al., 1996) of a synthetic NS4A peptide activator; these papers have recently been reviewed (Lemon, 1997; Kwong, 1998). This review will focus on the structure and function of the HCV NS3/4A serine protease and recent developments in the design of its inhibitors.

2. HCV infection and therapy

HCV is the major etiologic agent of transfusion-associated and community-acquired non-A, non-B hepatitis (NANBH) (Houghton et al., 1991; Houghton, 1996) with an estimated worldwide seroprevalence of approximately 1% (Purcell, 1994). Globally, there are an estimated 300 million carriers (Van der Poel, 1994) and there are 3.9 million infected individuals in the United States alone (Alter and Mast, 1994; Alter, 1997). Infection with HCV produces acute clinical hepatitis in approximately 20% of individuals. In most cases, HCV establishes a persistent infection resulting in chronic hepatitis and cirrhosis, which can lead to hepatocellular carcinoma (Colombo et al., 1989; Hollinger, 1990; Saito et al., 1990; Bukh et al., 1993; Houghton, 1996). The recent demonstrations that RNA transcribed from a functional HCV cDNA clone and intrahepatically injected into chimpanzees is infectious (Kolykhalov et al., 1997; Yanagi et al., 1997) and can cause hepatitis (Kolykhalov et al., 1997) has provided convincing evidence that infection with HCV alone is sufficient to cause the disease.

Current FDA-approved therapy for chronic infection with HCV includes treatment with interferon alpha (IFN-α) HCV (Hino et al., 1994; Tsubota et al., 1994; Main, 1995; reviewed in Linday (1997)) or combination therapy with ribavirin and IFN-α. Four forms of IFN-α have been evaluated for the treatment of chronic HCV disease, but no consensus has been reached on patient selection, dose, duration of treatment, or predictors of a sustained response to treatment (reviewed in Bhandari and Wright (1995), Sharara et al. (1996), Linday (1997)). Although approximately 35–50% of patients initially respond to IFN-α treatment, only a minority of patients ($\sim$8–20%) attain a sustained response (Hino et al.,

1994; Tsubota et al., 1994; Main, 1995). Recent clinical trials combining ribavirin and IFN therapy have reported a doubling of the sustained response rate over IFN monotherapy (Schalm et al., 1996; Reichard et al., 1998). The benefit of adding ribavirin is most pronounced in the case of IFN relapsers or non-responders where retreatment with IFN plus ribavirin increased the sustained virological response rate in 14–30% of patients (Brillanti et al., 1995; Schvarcz et al., 1995).

No vaccine against HCV is available. Two observations suggest that development of an effective vaccine will be difficult to achieve. First, studies of HCV infection in chimpanzees have revealed that no protective immunity is developed against superinfection with either homologous or heterologous strains of HCV (Farci et al., 1992). Second, the HCV virus is extremely variable and individuals are infected with 'quasi-species' of related but not identical viruses (Ogata et al., 1991) which provide the potential for HCV to evade immune surveillance by the formation of 'escape mutants' (Kato et al., 1993; Purcell, 1997; and reviewed in Major and Feinstone (1997)). The general usefulness of anti-viral therapies which inhibit the HCV NS3 protease will depend on their ability to inhibit proteases of different HCV subtypes. The ability of the HCV-H (1a) protease to process a HCV-BK (1b) substrate (Lin et al., 1994) suggests that essential element(s) of recognition may be conserved between different subtypes; thus development of broadly effective HCV NS3 serine protease inhibitors may be possible.

3. The structure of the HCV NS3 protease catalytic domain

3.1. Overall structural fold

Several years of intense research in the areas of molecular biology and biophysical chemistry finally led to the crystallization and X-ray structure determination of the NS3 protease domain in 1996. Love et al. (1996) reported the crystal structure at 2.4 Å resolution of residues 1–189 of the NS3 protease domain from the BK strain of HCV genotype 1b, while Kim et al. (1998) reported the 2.5 Å resolution structure of residues 2–180 of the NS3 protease domain from the H strain of HCV genotype 1a complexed to a peptide encompassing residues 21–36 of NS4A. These structures confirmed earlier predictions that the NS3 protease domain adopts a chymotrypsin-like fold and revealed new information about three potential targets within the protease domain for anti-viral design: (i) the enzyme active site, (ii) a structural zinc-binding site, and (iii) the NS4A binding site.

In the absence of a NS4A activator peptide, the NS3 protease domain folds into two structural sub-domains, each containing a six-stranded β-barrel, similar to the trypsin-like serine proteases. The enzyme active site lies in a cleft between the two sub-domains, with the N-terminal sub-domain contributing the histidine-57 and aspartic acid-81 of the catalytic triad, and the C-terminal sub-domain contributing the catalytic serine-139. The N-terminal 30 amino acids extend away from the rest of the protein in the structure published by Love et al. (1996), forming several β-strands that interact with hydrophobic surfaces on neighboring molecules in the crystallographic asymmetric unit.

In contrast, the binding of a peptide corresponding to the 21–39 amino acid core of NS4A causes the N-terminal sub-domain of NS3 protease to form an eight-stranded, distorted β-barrel, structurally similar to the N-terminal domain of chymotrypsin (Fig. 1). The NS4A peptide forms one strand of the β-barrel and is sandwiched between two β strands from the N-terminal sub-domain. This interaction results in a more tightly packed, less extended N-terminus for the protease domain, which buries an additional 2400 Å^2 of surface area. These structural observations are in good agreement with earlier deletion analyses of the protease domain which mapped the interaction site of NS4A to the N-terminus of NS3 (Bartenschlager et al., 1995b; Failla et al., 1995; Satoh et al., 1995). The extensive hydrophobic interactions between the NS4A activator peptide and the protease domain (Fig. 2) are consistent with previous studies which demonstrated that multiple hydrophobic residues in the central region of NS4A are essential for optimal

complex formation and activation of protease activity (Tanji et al., 1995; Lin and Rice, 1995; Butkiewicz et al., 1996; Shimizu et al., 1996).

3.2. Effect of NS4A on the conformation of the active site

The conformation of the catalytic triad and the oxyanion hole around the active site Ser-139 of the NS3 protease/NS4A peptide complex is very similar to that seen in other members of the trypsin family of serine proteases (Fig. 1). In contrast, the active site of the NS3 protease domain in the absence of NS4A deviates significantly from the normal catalytic triad configuration, in that the imidazole ring of His-57 is positioned too far away to effectively deprotonate the nucleophilic Ser-139 residue. Additionally, the Asp-81 side chain is oriented away from His-57, which makes it is unable to provide charge stabilization for the histidine during deprotonation of Ser-139. Thus, intercalation of NS4A into the N-terminal domain of NS3 appears to result in a spatial rearrangement of the active site towards the classical catalytic triad configuration, consistent with the observed increase in catalytic efficiency for the NS3/NS4A complex. This hypothesis is supported by biochemical and biophysical studies which showed that the binding of NS4A to NS3 primarily induces tertiary rather than secondary structure changes in the enzyme and changes the physicochemical requirements for optimal activity (Steinkuhler et al., 1996b; Bianchi et al., 1997).

3.3. Zinc-binding site

The HCV NS3 protease structure contains a zinc ion tetrahedrally coordinated by Cys-97, Cys-99, Cys-145, and, via an ordered water molecule, His-149. These residues are strictly conserved in all known HCV genotypes. The presence of a zinc

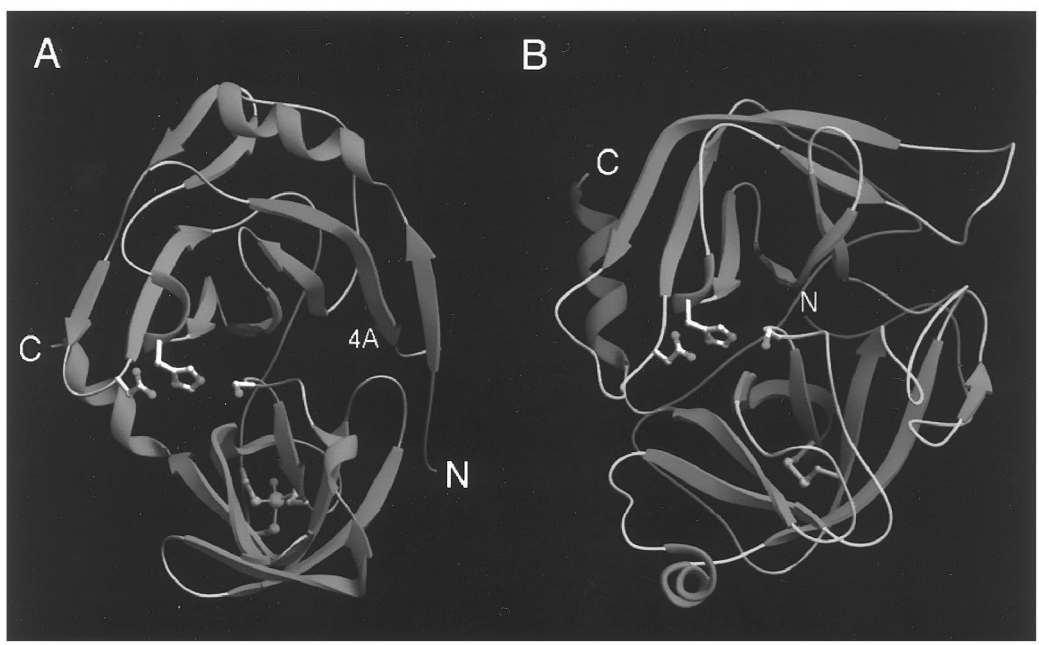

Fig. 1. Ribbon drawings of (A), the NS3 protease:NS4A peptide complex determined by Kim et al. (1996), and (B) chymotrypsin. The view from above the active site is approximately the same for each molecule. The Asp–His–Ser catalytic triad residues for each enzyme are displayed in ball-and-stick representation. The NS4A peptide is colored red. The bound zinc ion in NS3 protease is colored cyan and shown liganded to three cysteine side chains and one water molecule. The similarly located disulfide bridge in chymotrypsin is also shown. This figure was prepared using the program Ribbons vers. 2.0 (Carson, 1991).

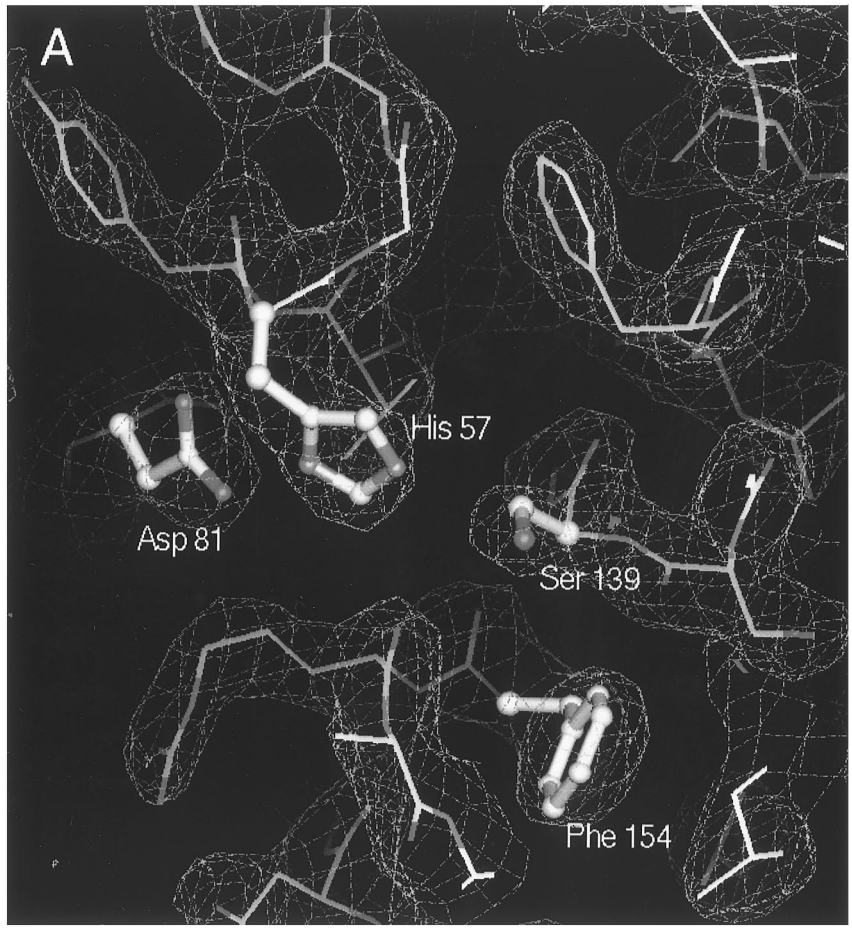

Fig. 2. Detailed views of the NS3 protease domain active site and NS4A binding site. (A) The catalytic triad residues Asp81, His57, and Ser139 are shown in ball-and-stick as is the Phe154 side chain which defines part of the substrate P1 binding pocket. Atoms are color coded by element type: carbon in white, nitrogen in blue, and oxygen in red. Experimental electron density is shown in magenta. (B) The NS4A peptide (yellow) lays between the two N-terminal β-strands of the protease domain, making extensive hydrophobic interactions. The NS3 protease domain is colored blue. This figure was prepared using the program Quanta 4.1 (Molecular Simulations).

binding site in the NS3 protease domain was previously predicted by homology modeling (De Francesco et al., 1996). Biochemical analyses revealed the presence of a tightly bound zinc ion which is present in equimolar ratio with NS3 and which is required for the generation of properly folded and active enzyme (De Francesco et al., 1996; Stempniak et al., 1997). Very weak inhibition was observed with the metal ion chelator EDTA (Lin and Rice, 1995; Kakiuchi et al., 1997) and with cupric ion (Hahm et al., 1995; Han et al., 1995; Kakiuchi et al., 1997). Both a slight enhancement (Han et al., 1995) and inhibition (Kakiuchi et al., 1997) on protease activity by the addition of zinc ion has been reported. The X-ray structures show that the zinc binding site is located at least 20 Å from the catalytic serine in the active site. This location is consistent with the zinc ion playing essentially a structural rather than catalytic role in the NS3/4A complex. Chymotrypsin contains a disulfide bond in a similar location to the zinc binding site in NS3 protease (Fig. 1), which may play an equivalent role in stabilizing the protein. Recently it has been sug-

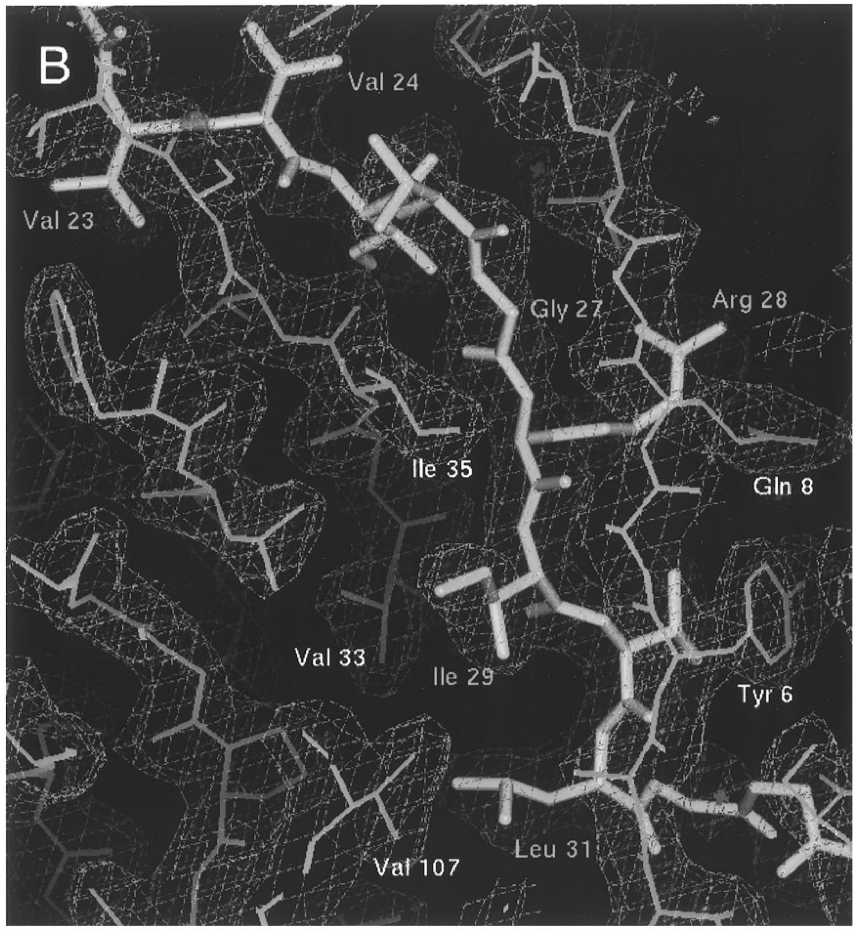

Fig. 2. (*Continued*)

gested that this zinc is the catalytic zinc of the NS2/NS3 metalloprotease that is required for cleavage between the NS2 and NS3 proteins (Wu et al., 1998).

Sequence alignment has revealed that the metal binding motif –C*X*C–...C*X*H, where *X* is any amino acid, is also present in the picornaviral chymotrypsin-like 2A cysteine protease (Bazan and Fletterick, 1988; Yu and Loyd, 1992). In addition, the presence of a tightly bound zinc, which is essential for structural integrity and stability, but not catalysis, has been observed in poliovirus and rhinovirus 2A proteases (Yu and Loyd, 1992; Sommergruber et al., 1994; Voss et al., 1995). The fact that this site is more conserved than the catalytic residues has led De Francesco et al. (1996) to propose a novel class of zinc binding chymotrypsin-like proteases and to suggest that the zinc-binding site is an attractive target for anti-viral therapy.

4. Model of a substrate docked into the active site

Besides the active site catalytic triad, the most prominent structural feature of the substrate binding site is the P1 specificity pocket, which is small, very hydrophobic, and constrained on the bottom by the flat aromatic ring of Phe-154 and on the sides by Ala-157 and Ile-135. The sulphydryl group of the substrate P1 cysteine is inserted into

the P1 pocket, which results in a favorable electrostatic interaction with the aromatic ring of Phe-154. This interaction was predicted by De Francesco et al. (1996) on the basis of a homology model of the NS3 protease active site docked with a peptide substrate and on 'specificity switching' mutations that were engineered into the P1 pocket of protease (Pizzi et al., 1994; Failla et al., 1996).

Our docking studies of a decapeptide substrate into the active site of NS3 protease derived from the crystal structure (Landro et al., 1997; Kim et al., 1998) confirmed the predicted interaction between the cysteine side-chain of the substrate P1 residue and Phe-154 (Fig. 3). In addition, the model suggests that the Tyr side-chain at P4′ of the substrate is in close proximity to Ile-29 of NS4A. This model is consistent with K_i data of peptide inhibitors measured in the presence and absence of NS4A (Landro et al., 1997). When NS4A was removed from the NS3:NS4A complex, the largest loss in binding was seen for inhibitors which extended to P4′. The NS3 protease/NS4A structure also suggests that NS4A makes substantial contacts with several loops on the prime side of the substrate binding channel. Removal of NS4A is likely to perturb these loops, which make contact with the P1′–P3′ residues of the modeled substrate. This is again consistent with experimental K_i data for inhibitors which extend to the prime side whose inhibition decreased by one order of magnitude upon removal of NS4A from the NS3 protease/NS4A complex (Landro et al., 1997). On the other hand, the K_i values of aldehyde inhibitors which bind only to the P side of the active site are not affected by the presence or absence of NS4A.

5. P and P′ substrate site requirements

A comparison of amino acid sequences of the four NS3-mediated cleavage sites (Table 1) reveals three conserved residues. Cysteine is conserved at the P1 position in the three *trans*-cleavage sites, while Thr occupies the P1 position of the *cis*-, NS3-4A cleavage site. A Ser at position P1′ is conserved in all NS3 cleavage sites except for the 4A-4B cleavage site of strain 1b, where it is replaced by Ala. In addition, an acidic residue is conserved at the P6 position of all cleavage sites, with either Asp or Glu present at this position.

In order to determine whether the conservation of P1, P1′ and P6 residues is due to a requirement for NS3 protease recognition and cleavage or to a structural requirement at the N- and C-termini of the resulting HCV nonstructural proteins, the effects of amino acid substitutions in the polyprotein substrate on NS3 protease activity have been examined. Some of these studies measured the kinetic parameters of NS3 protease acting on synthetic peptide substrates (Table 1) (Steinkuhler et al., 1996b; Landro et al., 1997; Urbani et al., 1997; Zhang et al., 1997), whereas others were performed on polyprotein substrates expressed in vitro or in mammalian cells (Kolykhalov et al., 1994; Bartenschlager et al., 1995a). The results demonstrate that the identity of the P1 residue is most severely restricted: even a mutation to Thr, found in the natural NS3-4A *cis*-cleavage site, abolishes NS3 cleavage in *trans*. (Urbani et al., 1997). These observations had been previously predicted by a model of substrate bound to NS3, which predicted the P1 residue to be small, hydrophobic, and capable of a favorable interaction with the aromatic ring of Phe-154 (Pizzi et al., 1994; Failla et al., 1995). The P1′ position can tolerate either Ser or Ala, but not a bulky aromatic residue (Urbani et al., 1997; Zhang et al., 1997). The observations is also consistent with the predicted structure of a NS3 protease/substrate peptide complex which describes a P1′ pocket that is not packed as tightly as the P1 pocket (Pizzi et al., 1994; Failla et al., 1995). In contrast to the P1 and P1′ positions, several substitutions of P6 Glu or Asp, including neutral and basic residues, are well tolerated by NS3 protease (Steinkuhler et al., 1996b; Urbani et al., 1997; Zhang et al., 1997). Clearly, the conservation of the P6 residue is due to a restriction other than the specificity of NS3 protease.

Whereas residues at P1 and P1′ are the most critical for substrate binding and cleavage by NS3 protease, substrate residues which are not conserved also contribute to the binding between NS3 protease and its substrate, as demonstrated by inverse alanine scanning of the substrate (Urbani

Table 1
The effect of amino acid substitutions in the substrate on catalytic efficiency of NS3 protease

Position	Cleavage site: 1a/1b				Tolerated: $(k_{cat}/K_m)_{mutant} = 0.01\text{-}1\ (k_{cat}/K_m)_{wt}$	Inhibiting: $(k_{cat}/K_m)_{mutant} < 0.1(k_{cat}/K_m)_{wt}$	Site	Reference
	3-4A	4A-4B	4B-5A	5A-5B				
P6	**D**	**D**	**E/D**	**E**	E,N	N-terminal delection	4A/4B-1b	(Steinkuhler et al., 1996b) (Urbani et al., 1997)
					A		5A/5B-1a	(Zhang et al., 1997)
P5	L	E	C	D	A		5A/5B-1a	(Zhang et al., 1997)
P4	E	M	T/S	V		A	5A/5B-1a	(Zhang et al., 1997)
P3	V	E	T	V	A		4A/AB-1b	(Urbani et al., 1997)
						A	5A/5B-1a	(Zhang et al., 1997)
P2	V	E	P	C	A		5A/5B-1a	(Zhang et al., 1997)
P1	T	C	C	C		T, V, A, P, F, S, G, L	4A/AB-1b	(Urbani et al., 1997)
						A,S	5A/5B-1a	(Zhang et al., 1997)
						T, A, V, L, Y, D	5A/5B-1a	(Landro et al., 1997)
P1′	S	S/A	S	S	S	F	4A/AB-1b	(Urbani et al., 1997)
					A		5A/5B-1a	(Zhang et al., 1997)
						F, W, K, A, D, Y, P	5A/5B-1a	(Landro et al., 1997)
P2′	T	Q/S	G	M	A		5A/5B-1a	(Zhang et al., 1997)
P3′	W	H	S	S	A		5A/5B-1a	(Zhang et al., 1997)
P4′	V	L	W	Y	A		5A/5B-1a	(Zhang et al., 1997)

Clevage site residues of strain 1b are only shown where they are different from the corresponding residues of strain 1a

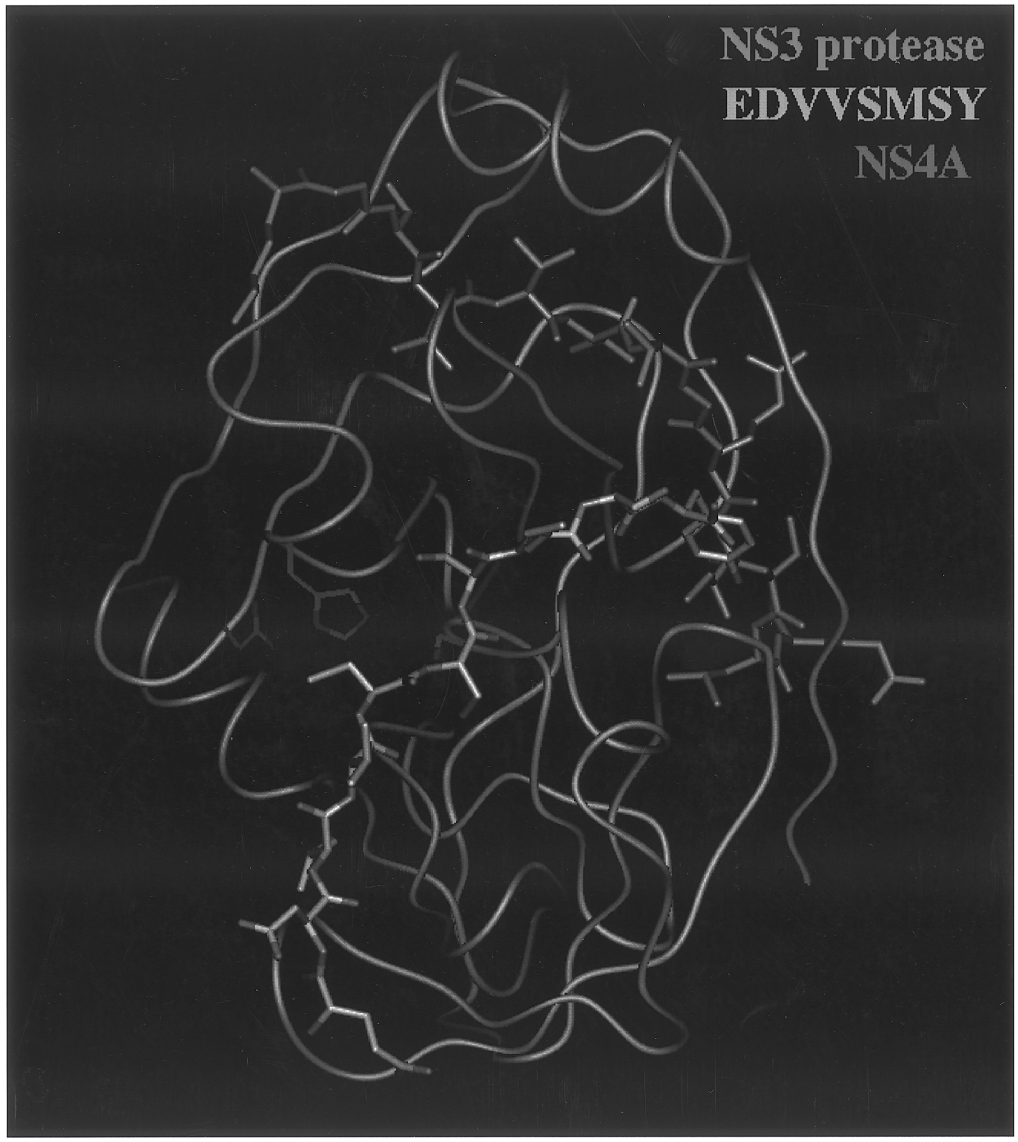

Fig. 3. Model of substrate (EDVVSMSY, color coded by atom type) bound to the active site of the NS3 protease (purple)–NS4A (blue) complex. The crystal structure of NS3/NS4A complex reported by (Kim et al., 1998) has been used to dock the substrate by the following procedure. The NS3 protease crystal structure was aligned with the crystal structure of α-chymotrypsin complex with eglin c (pdb code: 1acb (Frigerio et al., 1992) by using C_a atoms of the active site triad and the Arg155 (with equivalent triad residues and Ser-214 of 1acb, respectively) using the Quanta molecular modeling program. The model of the substrate was built into the NS3 active site guided by the bound conformation of the P4–P1′ segment of eglin c in the 1acb structure. The P6 and P5 residues were placed such that the amide and the carbonyl of P5 make hydrogen bonds with the carbonyl and the amide of Cys159 of NS3 protease, respectively. The P2′–P4′ was modeled to extend into the groove on the prime-side. The model was energy minimized in the active site of NS3/NS4A complex holding the enzyme atoms fixed.

et al., 1997). This study showed that wild-type residues at positions P6, P3, P1 and P1′, in the context of a polyalanine substrate, were sufficient to allow cleavage by NS3 protease, albeit at 80-

fold lower catalytic efficiency for the wild type substrate. The inclusion of wild type P4 residue into this substrate raised the catalytic efficiency to 25% of that of the wild type sequence.

6. The NS3/4A native complex

Although the N-terminal serine protease domain and the C-terminal NTPase/helicase domain of NS3 can be functionally separated, there is no evidence to suggest that the two domains are separated in vivo. Using NS3/4A purified from transiently transfected COS cells, Morgenstern and coworkers observed a five-fold stimulation of protease activity by poly(uridylic acid) [poly (U)] (Morgenstern et al., 1997). In contrast, poly (U) had no effect on the protease activity of the N-terminal protease domain complexed with a NS4A peptide. This observation suggests that binding of poly (U) to the C-terminal domain of NS3 influences the N-terminal protease domain. The physiological importance of this observation remains unclear. The determination of the structure of the native NS3/4A complex will likely reveal how each domain influences the fold of the other and affect each other's activities. In the absence of the determination of the native structure, it is interesting to examine how the activity of the full length (631 amino acids) NS3 protein complexed with the full length (54 amino acids) NS4A activator peptide differs from that of the full-length NS3 protein or the 181 residue N-terminal NS3 protease domain complexed with a 15–22 amino acid peptide corresponding to the core of NS4A.

As shown in Table 2, the catalytic efficiency reported in the literature for the NS3 protease domain on NS4A-4B and NS5A-5B peptide substrates ranges from 47 to 700 M^{-1} s^{-1} in the absence of NS4A activator peptide and from 484 to 20000 M^{-1} s^{-1} in the presence of NS4A activator peptide. The stimulatory effect of NS4A activator peptide on the catalytic efficiency of the protease domain ranges from 3- to 29-fold. The activity of the full-length NS3 is surprising low in the absence of NS4A activator peptide (5–32 M^{-1} s^{-1}) and is stimulated 56-fold to 278 M^{-1} s^{-1} with NS4A activator peptide. The wide range of reported catalytic efficiencies for the protease domain most likely reflects the exquisite sensitivity of the enzyme to its physicochemical environment, such as differences in pH, salt, and detergent composition of the assay conditions, difficulties in solubilizing the enzyme during purification, differences between 1a and 1b strain enzymes and substrates, and different degrees of saturation of NS3 protease with NS4A peptide. As shown in Table 2, the best optimized catalytic efficiencies reported are for the protease domain with NS4A activator peptide. In one case (Landro et al., 1997), the catalytic efficiency of NS3 protease domain exceeds that of the native NS3/4A complex. This suggests that under optimized conditions, the structural fold of the active site of the catalytic domain complexed with NS4A peptide is similar to that of the NS3/4A complex. Until the structure of the NS3/4A complex is solved, it is hard to determine the exact contribution of the N- and C-terminal ends of NS4A and the presence of the C-terminal helicase domain to the HCV NS3 protease conformation, activity and stability.

7. HCV NS3 protease high throughput assay development

The development of new anti-HCV protease inhibitors will be dependent on the expression and purification of large amounts of protein for biochemical characterization, structural studies and the screening of potential inhibitors for activity. High throughput primary screening using in vitro assays with purified protease and synthetic peptide substrates and structure-based drug design are currently being used in the search for inhibitors.

Early cell-free *trans*-processing assays for the determination of NS3 protease activity relied on the use of ^{35}S-radiolabelled substrates created through an in vitro transcription translation (IVTT) system. Proteins containing either the NS5A-5B, 4B-5A, or 4A-4B processing sites were cleaved in near stoichiometric amounts when they

Table 2
HCV NS3 and NS3/4A activity on peptide substrates

Form of NS3 (Expression System)		Form of NS4A	Substrate	K_m [μm]	k_{cat} [s^{-1}]	k_{cat}/K_m [M^{-1} s^{-1}]
Full length NS3/NS4A	$NS3_{1-631}/4A_{1-54}$ (Sf9) (Sali et al., 1998)	$NS4A_{1-54}$	5AB (P8-P8′K) DTEDVVCC↑SMSYTWTGK	59	0.220	3700
	$NS3_{1-631}/4A_{1-54}$ (Sf9) (Zhang et al., 1997)	$NS4A_{1-54}$	5AB (P8-P8′K) DTEDVVCC↑SMSYTWTGK	1.6	0.133	8300
Full length NS3	$NS3_{1-631}$ (*E. coli*) (Mori et al., 1997)	—	5AB (P10-P10′) GEAGDDIVPC↑SMSYTWTGAL	900	0.028	32
	MBP-$NS2_{126-200}$-$NS3_{1-589}$ (*E. coli*) (Shimizu et al., 1996)	—	5AB (P9-P9′) EAGDDIVPC↑SMSYTWTGA	411	0.002	5
		$NS4A_{18-40}$		108	0.030	278
NS3 Protease domain	$NS3_{1-180}$ (*E. coli*) (Steinkuhler et al., 1996b)	—	5AB (P6-P4′) EDVVCC↑SMSY	310	0.07	220
		$NS4A_{21-34}$		380	0.25	650
		—	4AB (P6-P4′) DEMEEC↑ASHL	100	0.005	47
		$NS4A_{21-34}$		43	0.023	574
	$NS3_{1-180}$ (*E. coli*) (Landro et al., 1997)	—	5AB (P5-P4′) EDVVαAbuC↑SMSY	270	0.18	700
		KK-$NS4A_{21-34}$		32	0.6	20 000
	$NS3_{1-188}$ (*E. coli*) (Mori et al., 1997)	—	5AB (P10-P10′) GEAGDDIVPC↑SMSYTWTGAL	310	0.26	839
	$NS3_{1-192}$ (*E. coli*) (Vishnuvardhan et al., 1997)	—	5AB (P10-P7′) GEAGDDIVPC↑SMSYTWT	250	0.033	132
		$NS4A_{15-34}$		99	0.26	2667
	$NS31_{13-201}$ (Sf9) (Steinkuhler et al., 1996a)	—	4AB (P10-P10′) YQEFDEMEEC↑ASHLPYIEQG	30	0.005	174
		$NS4A_{21-34}$		30	0.014	484

were mixed with or co-expressed with the NS3/NS4 complex or NS3 alone (Bouffard et al., 1995; D'Souza et al., 1995; Lin and Rice, 1995; Hamatake et al., 1996). As larger amounts of protease became available for study, a number of groups turned to HPLC cleavage assays of peptide substrates based upon these same sequences (Steinkuhler et al., 1996a; Kakiuchi et al., 1998). The use of such substrates gave quantitative information on the stimulatory effect of NS4A activator peptide on cleavage activity and the substrate specificity, including the strong requirement for Cys at P1, which had been predicted from a sequence analysis of the cleavage sites (Zhang et al., 1997). In addition, truncation data indicated a significant loss in binding upon deletion of the prime side residues from either the NS5A-5B (Landro et al., 1997) or 4A-4B sequences (Steinkuhler et al., 1996b). This loss is also reflected in attempts to utilize standard chromophoric substrates where the reporter group is released from the C-terminus of the scissile peptide bond. Ac-EDVV-Abu-C-pNA (based on the sequence of the NS5A-5B cleavage site) has been reported as a suitable substrate (Landro et al., 1997), however the K_m value for NS3/4A catalyzed cleavage is on the order of 1 mM and the k_{cat}/K_m value is only 200 as compared to the corresponding 5A/5B decapeptide with a K_m of 32 μM and a k_{cat}/K_m value of 20000 M^{-1} s^{-1}. It should be noted that kinetic parameters based upon the synthetic substrates for viral proteases are notoriously low; in vitro k_{cat}/K_m values range no higher than 400 and 50 M^{-1} s^{-1} for human cytomegalovirus (Sardana et al., 1994) and HSV protease (Darke et al., 1994), respectively.

Nevertheless, recent reports of advances in high throughput assays based upon peptide substrates spanning the P6–P4′ subsites (and beyond) have appeared. Sudo et al. (1996) report the use of an N-dansylated 20-mer based upon the NS5A-5B cleavage site as a substrate for a sensitive HPLC assay. Proline was substituted for cysteine at P2 to prevent the formation of an intramolecular disulfide linkage with the preferred P1 Cys. Using a maltose binding protein-NS3-NS4A fusion protein as the enzyme, the authors reported a K_m value of 69 μM; a k_{cat}/K_m value of 4000 could be estimated from the reported data.

An ELISA based assay of cleavage activity was reported by the same group (Takeshita et al., 1997). A C-terminal biotinylated 17-mer also constructed from the NS5A-5B cleavage site and containing P2 proline was employed. After NS3 catalyzed proteolysis, the P1 cysteine was acetylated with iodoacetate, and the N-terminus of the cleaved peptide was allowed to react with *N*-hydroxysuccinimide-digoxigenin. The labeling reaction was quenched with glycine and the solution transferred to streptavidin coated microtiter plates, followed by a washing step. The addition of an anti-digoxigenin-alkaline phosphatase antibody and subsequent visualization with *p*-nitrophenyl phosphate gave a colormetric quantitation of the amount of cleaved peptide. The absorbance/product relationship was linear in the range of 0.1–10 ng/well, and the sensitivity was roughly equal to that of HPLC based methods. This assay has the advantage of being automated to 96-well format; however, the authors noted that both strict temperature control and thorough mixing of the small volumes (5 μl) were necessary for reproducible results. For the analysis of a large number of samples, the cost of reagents also becomes an important factor.

Bianchi et al. (1996) reported that k_{cat}/K_m values for NS3/4A cleavage of peptide based substrates could be substantially improved by replacing the scissile amide bond with an ester linkage. Suitable depsipeptide substrates with a P1 Cys proved to be difficult to synthesize, likely due to potential problems with intramolecular cleavage of the ester bond by an adjacent nucleophile. On the other hand, the replacement of P1 with either Abu or Thr on depsipeptide dodecamer based on the NS4A-4B cleavage site could be processed efficiently by the NS3/4A complex as analyzed by HPLC. A K_m value of 11 μM with k_{cat}/K_m or 14000 M^{-1} s^{-1} was reported for the P1 Abu peptide.

Taliani et al. (1997) then utilized a depsipeptide for the development of a fluorogenic assay based upon resonance energy transfer. As before, a P1 Abu substitution was used; in addition the peptide contained a fluorescent donor 5-[(2′-aminoethyl)

amino]naphthalene sulfonic acid (EDANS), and an acceptor group 4-[[4′-(dimethylamino)phenyl] azo]benzoic acid (DABCYL). Intact, the fluorescence of the donor is intramolecularly quenched by the acceptor through resonance energy transfer. As the peptide is cleaved, the quencher is no longer attached, and the fluorescence of the donor generates a suitable signal. The best substrate synthesized, Ac-DED(EDANS)EEAbuY[COO] ASK(DABCYL) exhibited a K_m value of 4.3 μM and a k_{cat}/K_m value of 340000 M^{-1} s^{-1} for NS3/4A catalyzed cleavage. This value is among the highest reported to date. The detection limit of NS3 was reported to be 3 nM, with the detection time of 1 min. A peptide based inhibitor was used to demonstrate the suitability of this assay for the determination of IC_{50} values. The high catalytic efficiency with this substrate allows for the use of minimal enzyme concentrations, which would be required for screening compounds in nanomolar amounts as found in some combinatorial libraries or natural product collections. The fluorescent signal contributes to the high sensitivity of the assay, but precautions need be observed so that inhibitor quenching of the signal does not contribute to false positives in the assay. Within these caveats, the substrate design improvements of exchanging the amide linkage and the incorporation of suitable fluorescent reporter groups without loss of binding potency have resulted in an assay highly suitable for the rapid screening of potential HCV protease inhibitors.

8. Inhibitors of HCV NS3 protease

Because the substrate binding pocket is shallow, relatively solvent-exposed and non-polar in nature, with beta sheets lining both sides of the groove, the binding of small molecule inhibitors is quite inefficient. This explains why previous biochemical searches for a good lead inhibitor structure starting from known protease inhibitors were unsuccessful. Biochemical studies with known protease inhibitors had revealed that the NS3/4A protease is inhibited by high concentrations of chymotrypsin-like serine protease inhibitors such as *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), chymostatin, and Pefabloc SC, or by general serine protease inhibitors such as 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (D'Souza et al., 1995; Hahm et al., 1995; Lin and Rice, 1995; Markland et al., 1997). This is in agreement with the overall similarity in fold of NS3 protease to chymotrypsin. Little or no inhibition was observed with other classical serine protease inhibitors such as diisopropyl fluorophosphate (DIP), 3,4-dichloroisocoumarin, aprotinin, antipain, leupeptin, and *N*-α-ρ-tosyl-L-lysine chloromethyl ketone (TLCK) (Bouffard et al., 1995; D'Souza et al., 1995; Hahm et al., 1995; Kakiuchi et al., 1997). No inhibition was observed with other standard cysteine, aspartic, and metalloprotease inhibitors such as phosphoramidon.

The observation that the addition of high concentrations of conventional serine protease inhibitors resulted in either no inhibition or very weak inhibition of the NS3 protease suggests that the active site of the protease is different from that of cellular serine proteases and that specific non-toxic inhibitors of NS3 could be developed. This is further supported by the observation that, unlike serine proteases such as trypsin and chymotrypsin, the NS3 protease cannot cleave small peptide substrates. Truncation analyses of peptide substrates have shown that the minimal length for efficient cleavage by NS3 protease is a decamer spanning residues P6–P4′ (Steinkuhler et al., 1996b). The major effect of C- and N-terminal truncations was an increase in the K_m values, indicating that hydrophobic and electrostatic interactions between residues lining the shallow binding pocket outside of the active site and substrate residues distal to the scissile bond play a major role in contributing to the binding efficiency of the substrate. Thus, the binding of substrate in the NS3 protease active site is stabilized by at least three interactions: the cysteine electrostatic interaction with the phenylalanine in the bottom of the P1 site, the hydrogen-bonding interaction between the long extended substrate and the two adjacent beta strands of NS3 in the substrate binding groove and the hydrophobic effect of the complementary hydrophobic surfaces. Prime and nonprime truncation analysis of peptidyl inhibitors containing the P1′ residue tetrahydroisoquinoline-3-carboxylic acid (Tic)

derived from the NS5A/B cleavage site supported a model where most of the binding energy for a given peptidyl inhibitor was extracted from the prime side by the NS3 protease catalytic domain (Landro et al., 1997). In addition, a large increase in affinity of the Tic-inhibitor for the protease was observed in the presence of NS4A activator peptide; modeling of this interaction suggests that the P4′ residue is in close proximity or in direct contact with NS4A. This is consistent with a large loss in binding of inhibitors which extend to P4′ upon removal of NS4A. Because of the difficulties of de novo design of non-toxic small molecule inhibitors to bind to the NS3/4A protease substrate binding pocket, many groups have turned to high throughput screening of chemical and natural product libraries to search for novel lead molecules. Chu et al. (1996) reported the identification of a phenatrenequinone compound as a secondary metabolite in a *Streptomyces* fermentation culture with an IC_{50} of 7 μM in an in vitro assay for HCV protease. Random screening of chemical libraries by Sudo et al. (1997b) resulted in the identification of 2,4,6-trihydroxy,3-nitrobenzamide (THNB). The most potent in a series of 12 THNB derivatives, RD3-4082 had an IC_{50} of 5.8 and 3 μM in the presence and absence of NS4A, respectively. Unfortunately, most of the THNB derivatives tested also inhibited human serine proteases such as chymotrypsin and elastase, making them inappropriate candidates for clinical use. Sudo et al. (1997a) also described a series of nine thiazolidine compounds, some of which were claimed to inhibit HCV protease in a noncompetitive manner. Unfortunately, most of these compounds also showed inhibitory activity against other human serine proteases such as chymotrypsin, trypsin, plasmin, and elastase. Kakiuchi et al. (1998) screened a chemical library of 2000 compounds and identified two halogenated benzanilide inhibitors with IC_{50}s < 10 μM against HCV protease, which did not inhibit other serine proteases in the same concentration range. Some groups have turned to screening of antibody fragments (Martin et al., 1997) and phage-displayed libraries of 'minimized' antibody-like protein for NS3 protease inhibitors (DiMasi et al., 1997).

9. Bioassays for NS3 protease activity and HCV anti-viral assays

The development of secondary in vivo bioassays to test whether an inhibitor can function in a cellular environment is a critical step in the drug development process. Ideally, one would test whether a potential NS3/4A protease inhibitor inhibits HCV replication in cultured cells infected with HCV. Unfortunately, the testing of candidate anti-viral molecules has been limited by the lack of a robust in vitro system for growing HCV in cultured cells. In the systems described to date, the level of HCV RNA replication is so low that RT-PCR is required for detection of the replicating viral genomes reviewed in (Bartenschlager, 1997; Major and Feinstone, 1997).

Two cell-based systems have been described for testing the activity of NS3 protease inhibitors. Hirowatari et al. (1995) have developed a system in mammalian cells in which NS3 protease cleavage is required for transcription of a reporter gene. Conversely, Song et al. (1996) developed a system in yeast in which NS3 protease cleavage shuts down transcription of a reporter gene. Because of the low fidelity of RNA-dependent RNA polymerases, the development of resistance to anti-viral drugs will probably become a major hurdle in the small-molecule anti-viral therapy for HCV as has been the case for anti-HIV therapy. The development of chimeric sindbis virus (Cho et al., 1997; Filocamo et al., 1997) and chimeric polio virus (Hahm et al., 1996) whose viral replication is dependent on NS3 protease activity will allow investigators to study the development of NS3 protease inhibitor resistance.

10. Conclusion

Major advances have been made in elucidating the structure and function of the HCV NS3 serine protease domain and its NS4A activator peptide. The driving force behind most of this work is the effort to develop NS3/4A protease inhibitors which would serve as broadly effective therapies for infection with HCV. Potential targets for the development of anti-viral agents in the N-termi-

nus of the NS3/4A complex include the serine protease active site, the structural zinc binding site, and the NS3-NS4A interface. The structure of the NS3 C-terminal helicase domain has been recently determined (Kim et al., 1995; Yao et al., 1997); this domain contains further potential targets for anti-viral agents (inhibition of NTPase, unwinding, and RNA binding activity). It is important to keep in mind that the NTPase/helicase domain and the 54 residue NS4A activator are present in the full-length NS3/4A protease expressed in HCV infected cells. These additional elements may well affect the structure and function of the NS3 serine protease. A crystal structure of the full-length NS3/4A is needed to elucidate the role that the C-terminal domain of NS3 and the full-length NS4A play in NS3 protease activity and its implications for inhibitor design.

Acknowledgements

We thank John A. Thomson for his critical review of the manuscript.

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