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Chapter 542

Hepatitis A Virus Picornain 3C

Databanks

MEROPS name: hepatitis A virus-type picornain 3C
MEROPS classification: clan PA, subclan PA(C), family C3, subfamily C3E, peptidase C03.005
Tertiary structure: Available
Species distribution: known only from hepatitis A virus
Reference sequence from: hepatitis A virus (UniProt: P14553)

Name and History

The autoproteolytic activity of the hepatitis A virus (HAV) polyprotein and the proteolytic activity of the HAV 3C gene product were initially inferred from the similarity of the sequence of the HAV RNA genome to those of other picornaviruses [1]. The 3C gene product of picornaviruses is commonly referred to as the 3C proteinase [2]. Shortly after the name picornain 3C was introduced via recommendation by the IUBMB.

Activity and Specificity

Several different peptide-based assays of the proteolytic activity of HAV 3C have been reported [3–5]. Malcolm *et al.* [4] used a discontinuous trinitrobenzene sulfonate assay of free amino groups to measure the activity of HAV 3C on a peptide substrate acetyl-Glu-Leu-Arg-Thr-Gln↓Ser-Phe-Ser-NH2. This peptide sequence was derived from the 2B/2C cleavage site of the HAV polyprotein. Jewell *et al.* [3] developed a continuous fluorescence quench assay with dansylated peptides as substrates. Schultheiss *et al.* [5] separated and quantified the amounts of the cleaved peptidyl products on a reverse phase HPLC. The pH optimum of the proteolytic activity lies between pH 7.0 to 8.5 and the optimal peptide substrates of HAV 3C require residues from P4 to P2′ positions [3].

It is noteworthy to mention that the natural substrates of HAV 3C and related viral cysteine/serine proteinases are either translating or nascent viral polyprotein as well as processing precursors. Therefore, *in vitro* peptide-based assays have their limitations in that they do not completely mimic the native proteolytic events occurring in an infected cell. Although Schultheiss used *in vitro* translated segments of viral polyprotein encompassing cleavage sites as HAV 3C substrates, the accurate quantification of such reaction was difficult and cumbersome for kinetic studies [5,6]. On this front, a recent attempt to address such deficiency was reported by Huitema & Eltis [7] in which a linker peptide sequence carrying the cleavage site for HAV 3C was inserted between two versions of modified yellow fluorescent proteins that are capable of Förster resonance energy transfer (FRET). Cleavage in the linker sequence is accompanied by the concomitant loss of FRET signal. Albeit the potential adaptability in turning this assay into a high-throughput screening vehicle, the utility of this method, however, is also limited as it cannot study the influence of other factors on proteolysis such as putative exosites.

The substrate sequence of the 3C cleavage sites in the HAV polyprotein were initially predicted from sequence homology of the HAV genome to the other picornaviral genomes [1]. The location of several cleavage sites has subsequently been confirmed or corrected experimentally [5,8–13]. Table 542.1 shows the amino acid sequence of seven HAV 3C cleavage sites in the polyprotein which have been experimentally confirmed.

Jewell *et al.* [3] and Petithory *et al.* [14] determined the sequence preferences of HAV 3C cleavages on peptide substrates. HAV 3C requires a Gln in the P1 position of a substrate and has a strong preference for a hydrophobic residue in P4. In addition, all the natural cleavage sites in the viral polyprotein have either Ser or Thr in P2 (Table 542.1).

HAV 3C is inhibited by typical Cys modifying agents such as N-methylmaleimide and iodoacetamide. Some inhibitors of chymotrypsin-like serine proteinases, such as TPCK, are also effective against the enzyme [4]. Other more specific, synthetic inhibitors against HAV 3C including both peptidyl and nonpeptidyl compounds have also been developed [6,15–20]. Malcolm *et al.* determined quantitatively the inhibition of HAV 3C with peptide substrate-derived aldehyde inhibitors in which the basic design of the inhibitor is to have a reactive 'warhead' appended C-terminally to a tetrapeptide analog

TABLE 542.1 Hepatitis A virus 3C proteinase cleavage sites in the viral polyprotein

Cleavage sites (viral gene products)	Amino acid sequences							
	P5	P4	P3	P2	P1	P1′	P2′	P3′
1B/1C (VP2/VP3)	Pro	Leu	Ser	Thr	Gln	Met	Met	Arg
1C1D (VP3VP1)	Asp	Val	Thr	Thr	Gln	Val	Gly	Asp
2A2B	Gly	Leu	Phe	Ser	Gln	Ala	Lys	Ile
2B2C	Glu	Leu	Arg	Thr	Gln	Ser	Phe	Ser
2C3A	Glu	Leu	Trp	Ser	Gln	Gly	Ile	Ser
3B3C	Pro	Val	Glu	Ser	Gln	Ser	Thr	Leu
3C3D	Lys	Ile	Glu	Ser	Gln	Arg	Ile	Met

representing the P4-P3-P2-P1(Q^{dm}) residues of a substrate with P1(Q^{dm}) being glutaminal with its side chain amide dimethylated [21]. This design was followed by other experimentations that saw the replacement of the aldehyde group by halomethyl ketone or phthalhydrazide [6,16]; in one variant of the latter case, the P1 Gln was also substituted with a 2-oxo-pyrrolidine ring to improve the inhibitory effect [18]. Similarly, Huang *et al.* [20] described several series of compounds each carrying hydrazo-*o*-nitrophenylsulfenamide (HNPS), frame-shifted hydrazo-*o*-nitrophenylsulfenamide (FSHNPS), azaglutamine sulfonamide (AS) and haloacetyl azaglutamine (HA) as potential active site sulfur-reacting groups [20]. When tested on HAV 3C peptidase activity, HNPS showed little inhibitory effect, AS worked as weak competitive inhibitor, whereas both FSHNPS and HA irreversibly modified the catalytic Cys172 residue through disulfide bridge and alkylation, respectively. Two major groups of nonpeptidic inhibitors against HAV 3C were developed [17,19,22]. One group relies on a β-lactone moiety, which covalently modifies the nucleophilic S^{γ} of the catalytic Cys172 and by serendipity, the imidazole ring of a surface residue His102. Although both reactions can occur in solution, only the species with single modification at His102 was crystallized [23]. As it turned out in the crystal structure, the covalent attachment of the inhibitor (N-carboxybenzyl serine β-lactone) at His102 is crucial for crystal packing: the benzyl group is ideally inserted into a hydrophobic pocket located on the surface of a neighboring molecule in the crystal (see Figure 542.1). The second group of inhibitors derives its design from a pyridinyl thiophene ester, selected through a high-throughput screening of 50 000 compounds [17,24]. Some of the inhibitors in this group reached IC_{50} as good as 50 nm against HAV 3C; however, the inactivated enzyme slowly hydrolyzes the covalent acyl-enzyme adduct and regains proteolytic activity [25]. The ester function in the inhibitors was found to be indispensable for its activity against HAV 3C and a related viral enzyme, the SARS-CoV 3C-like enzyme. In essence, this class of inhibitors works like competitive substrate analogs that showed much higher affinity to the enzyme than peptides with more than 500-fold lower Km [25].

Structural Chemistry

HAV 3C is a single chain of 219 residues with a relative molecular mass of 24 093. The purified enzyme from a bacterial expression system contains only the first 217 residues [4]. The first crystal structure obtained for a picornain was the structure of an inactive mutant of the HAV 3C enzyme [26]. This was followed by the publication of several refined crystal structures of proteolytically competent HAV 3C, either in *apo* form or in complex with various inhibitors [23,27,28]. The overall fold and domain structure of the HAV 3C picornain resembles that of the chymotrypsin-like serine proteinases (Clan S1) with the proteolytic active site formed between two anti-parallel β-barrel domains (Figure 542.1).

Unique features of the HAV 3C picornain are the amino- and carboxyl-terminal helices that pack against the opposite domain, and a long anti-parallel β-ribbon that extends from the β-barrel of the carboxyl-terminal domain and forms part of the active site (colored green in Figure 542.1A). Cys172, His44 and Asp84 form the canonical catalytic triad in the active site. In the first refined crystal structure of the active enzyme, an ordered water molecule takes up the place of the carboxylate of a third member of a typical catalytic triad. It was thus suggested that a charged form of the side chain of Tyr143 stabilizes this arrangement and may be involved in catalysis [28]. However, more recent, higher-resolution crystal structure of HAV 3C in a different crystal form confirmed the existence of the canonical Cys:His:Asp catalytic triad in the enzyme's active site, finally laying the 'dyad *vs.* triad' uncertainty to rest [23]. In the newer structural model, the side chain carboxylate of Asp84 interacts with the imidazole ring of His44 in ideal distance and orientation to stabilize the developing charge in His44 during catalysis. Besides the catalytic residues, the S1 specificity-determining residue is also highly conserved among the picornains. His191 lies at the bottom of the S1 specificity pocket and is in a position to interact with the oxygen of the carbonylamide of a Gln residue in the P1 position of a substrate. There is no partner for an interaction with the side chain amide of the P1 substrate Gln in the crystal structure. This is in agreement with the finding that a peptide substrate with NN-dimethyl glutamine in

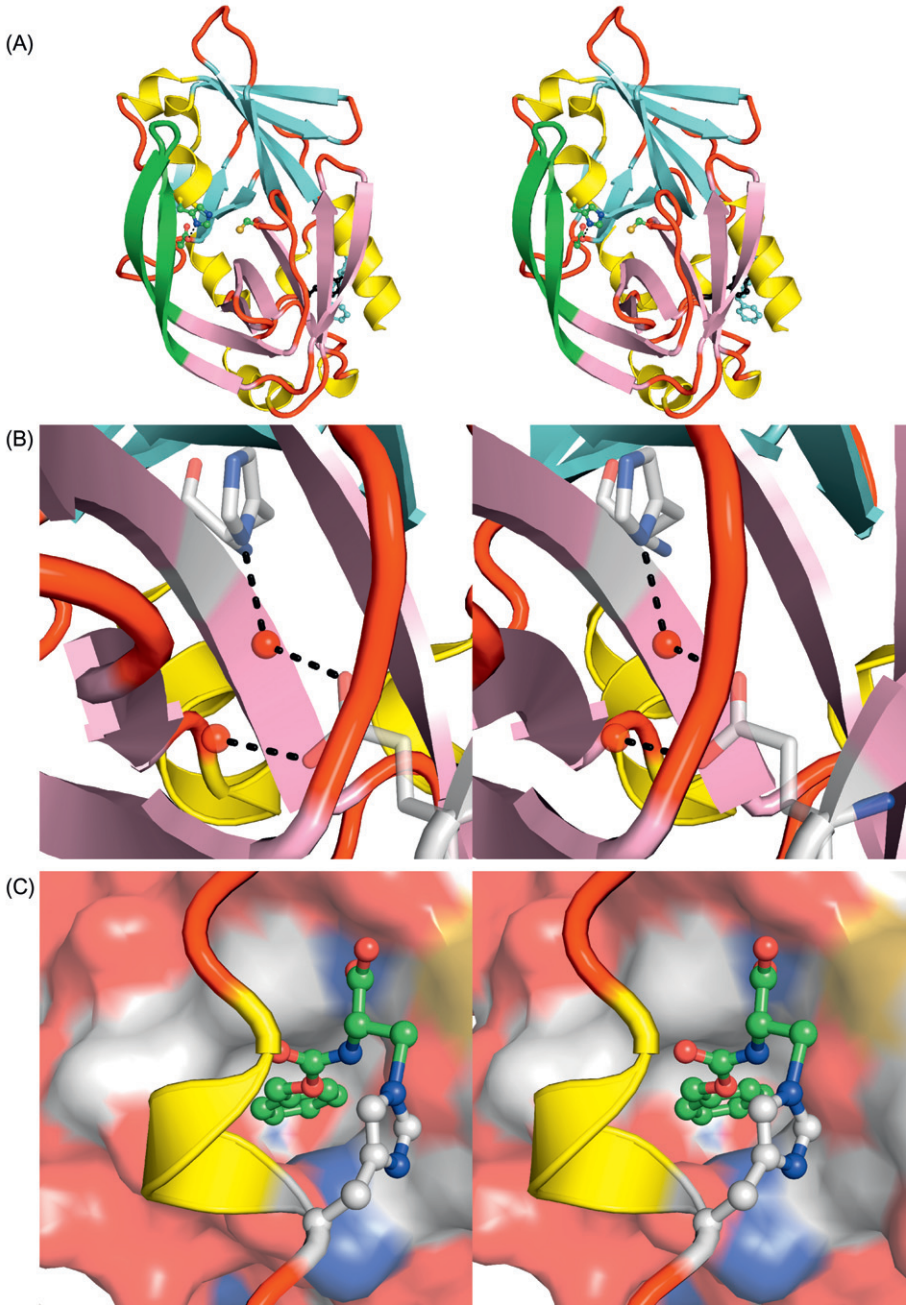

FIGURE 542.1 Stereo views of a cartoon diagram showing several important structural features of HAV 3C picornain. (A) Overview of the crystal structure. The N- and C-terminal β-barrels are colored cyan and pink, respectively. The β-hairpin extended from the C-terminal β-barrel is colored green. The helices and loops are colored gold and red, respectively. The catalytic triad, Asp84, His44 and Cys172, are shown in ball-and-sticks mode and colored by atom type (carbon, green; oxygen, red; nitrogen, blue; sulfur, yellow). Hydrogen bond is depicted in dashed line. (B) The buried side chain atoms of Glu132 form a water-bridged interaction with His191, the S1-specificity residue. The carbon atoms of His191 and Glu132 are colored white. Oxygen and nitrogen atoms are colored red and blue, respectively. Hydrogen bonds are shown in black dashed lines. (C) The covalent linkage between N-Cbz serine β-lactone (ball-and-stick model with carbon atoms colored green) to His102 (ball-and-stick model with carbon atoms colored gray) and its binding to a hydrophobic pocket on the surface of a neighboring molecule (surface representation with carbon atoms shown in gray).

the P1 position is equally well hydrolyzed [15]. From the crystal structure, it seemed possible to suggest that the interaction of His191 with a buried, and presumably uncharged, Glu132 via buried solvent (water bridge) is responsible for the selectivity for Gln in the P1 position of a substrate (Figure 542.1B).

It may be worth mentioning the crystal structure of HAV 3C in complex with a peptidyl inhibitor carrying a phthalhydrazine headgroup as 'warhead'. This inhibitor was initially designed to function as a non-covalent inhibitor for 3C enzymes as phthalhydrazine is considered a poor leaving group. However, the crystal structure surprisingly revealed a covalent linkage formed between the inhibitor and the active site cysteine residue. The phthalhydrazine headgroup of the inhibitor was displaced by the S^{γ} of Cys172 of HAV 3C as the result of a nucleophilic attack by the enzyme. Repeating the *in vitro* proteinase activity assay confirmed that the inhibitory effect is slow-acting requiring hours of pre-incubation of the compound with the enzyme but nevertheless irreversible. It was also derived from this structure that an unusual episulfide cation may be the intermediate molecular species that is

formed during the chemical reactions leading to either inhibition or peptide hydrolysis [27].

Preparation

HAV 3C picornain has been expressed in bacteria [4,9,29], cell free transcription-translation systems [10,30] and eukaryotic cells [12,13]. For kinetic and structural studies the enzyme has been purified from a bacterial overexpression system as described by Malcolm *et al.* [4].

Biological Aspects

Due to the protracted replication cycle of HAV and the low yield of viral gene products in cell culture, the study of all aspects of viral replication, including polyprotein processing, is difficult in HAV [31]. Furthermore, HAV infection does not result in inhibition of host cell protein synthesis as do the infections by many other picornaviruses. Therefore, investigations of HAV polyprotein processing rely on expression of cDNA constructs of the HAV genome in *in vitro* and *in vivo* systems. Interpretation of these results is further complicated by the appearance of aberrant initiation and premature termination products [11]. Schultheiss *et al.* [5] found a differential dependence on enzyme concentration for the 3C-mediated cleavages of the HAV polyprotein. How well this reflects the order of events during viral polyprotein processing *in vivo* is not clear. Nevertheless, it is becoming increasingly obvious that HAV is distinct from the other members of the *Picornaviridae* viral family in its polyprotein processing [5,12,13]. Most importantly, 3C appears to be the only virally encoded entity that shows proteolytic activity in HAV [5]. This is in sharp contrast to other picornaviruses which typically have two functional viral proteinases.

Picornaviral genomes are generally only ~7500 nucleotides long and encode less than a dozen individual viral proteins when fully processed from polyproteins. Such genetic austerity is compensated via several means to enhance the coding capacity of their RNA genomes, one of which is to have individual protein engaged in two or more different tasks. Besides their role in polyprotein processing, 3C proteinases are also involved in viral mRNA translation, viral genomic RNA replication and virus-host antagonism [32]. For example, 3C or its precursor, 3CD, was found to specifically enhance the uridylylation of VPg, the protein primer of polioviral RNA synthesis [33,34]. Aphthoviral 3C proteinase cleaves eIF4G to both inhibit host cellular translation and enhance viral IRES-directed translation using the C-terminal fragment of eIF4G [35]. Polioviral 3C is also capable of hydrolyzing other cellular factors that are critical for host mRNA translation or gene transcription, e.g., La autoantigen, PTB, PABP, TBP and TFIIIC, all contributing to shutting down host cellular protein production during viral infection [36–39]. HAV 3C is found to cleave PABP, PTB, and PCBP2, but not La [40–43]. The consequence of all these cleavage events invariably led to reduced viral RNA translation. Even the non-cleavage of La has the same effect because full-length La has inhibitory effect on HAV viral translation. It has been hypothesized that the relatively inefficient protein production in HAV may be the cause of its rather unique evolution and pathogenesis profile among picornaviruses: it survives by not eliciting as strong a cellular antiviral response as the other members during an infection [44].

The RNA binding activity of HAV 3C with affinity towards the 5′ untranslated region of the viral genome was initially evidenced by an *in vitro* study [45]. Crystal structures reveal that a 5-residue motif (K^{95}FRDI^{99} in HAV 3C), highly conserved throughout the 3Cs of all picornaviruses, forms a defined RNA binding site on the surface of the molecule more than 20 Å away and opposite from the proteolytic active site [23,28]. In other picornaviruses, the RNA recognition activity of 3C has been implicated in the initiation, organization and regulation of viral RNA replication but this has not been investigated in HAV except in some *in vitro* work [45]. Interestingly, in the crystal structure of HAV 3C in complex with a β-lactone serine inhibitor, the RNA-binding motif is in close proximity to a surface hydrophobic pocket where the inhibitor covalently modifies the enzyme [23]. A study by Peters *et al.* [46] indicated that dimerization of HAV 3C enhances its association with RNA while reducing its proteolytic activity and that binding of substrate and RNA to monomeric HAV 3C is cooperative. Taken together, these two reports led to a tempting hypothesis that perturbing the RNA-binding property of HAV 3C may in turn affect its proteolytic activity.

Employing various processing precursors in various tasks during viral infection is yet another major way of maximizing the coding capacity of picornaviral genomes. It has been well established that in several picornaviruses, *e.g.* poliovirus and rhinovirus, 3CD, the precursor of 3C, instead of 3C, is the main viral proteinase. Furthermore, their 3CD proteins showed distinct properties such as RNA binding and interacting with host proteins that are critical in the synthesis of viral RNA. While 3CD does not have a proteolytic role in the HAV polyprotein processing, 3ABC seems to be able to cleave both viral polypeptides and cellular factors such as MAVS to turn off mitochondria-dependent antiviral defense mechanism [47]. Moreover, HAV 3ABC binds to both the 5′ and 3′ nontranslated regions of viral RNA with much higher affinity than either 3AB or 3C, implicating the role of

3ABC in viral replication and perhaps the biological reason for HAV 3ABC's relative stability *vs*. the 3ABC precursor in other picornaviruses [48].

Distinguishing Features

The 3C picornain from HAV is larger than the 3C gene products from other picornaviruses (219 residues *vs*. 183 in rhino- or poliovirus) [2]. As evidenced by crystal structures, HAV 3C also boasts the longest β-hairpin structural motif among picornain structures known to date that forms part of the substrate binding site (Figure 542.1A). Antisera specifically directed against HAV 3C have been described [8,12,30].

Related Peptidases

Other than those mentioned in other chapters of this handbook, the 3C proteinases have quite a widely spread presence in insect viruses, *e.g*. acute bee paralysis virus, Kashmir bee virus, Black queen cell virus, Plautia stali intestine virus, Himetobi P virus, *etc*. Most of these viruses belong to the family of *dicistroviridae*.

Further Reading

Kusov *et al.* [49] described the evolutionary strategy used by HAV with an emphasis on its codon choice. Coleman *et al.* [50] re-discovered the concept of codon pair bias in biology and applied it to the analysis of viral genomes.

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