CHAPTER 7

The 3C Proteinases of Picornaviruses and Other Positive-Sense, Single-Stranded RNA Viruses

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A. Introduction

Picornaviruses are a family of viruses which belong to the large group of positive-sense, single-stranded RNA viruses (Rueckert 1996). It was realized 30 years ago that the product of the translation of the RNA genome of these viruses is proteolytically processed to yield the mature viral proteins (Summers and Maizel 1968; Korant 1972). Subsequently, it could be shown for two different picornaviruses that the processing enzyme is a specific, virally encoded proteinase (Pelham 1978; Gorbalenya et al. 1979; Korant et al. 1979; Palmenberg et al. 1979). Once the amino-acid sequences of the viral proteinases became available, predictions were made concerning the structure of the picornaviral 3C proteinases (Grobalenya et al. 1986; Bazan and Fletterick 1988; Gorbalenya et al. 1989). These predictions were remarkable. Based on an analysis of several conserved sequence motifs within the amino-acid sequence of the 3C proteinases, it was suggested that these proteinases are structurally related to the chymotrypsin-like proteinases but with a cysteine residue as the active-site nucleophile. Crystal structures of 3C proteinases from two picornaviruses confirmed this prediction (Allaire et al. 1994; Matthews et al. 1994). At present, crystal structures of 3C proteinases from viruses, belonging to three different genera of the picornaviruses, have been published (Matthews et al. 1994; Bergmann et al. 1997; Mosimann et al. 1997).

The chymotrypsin-like cysteine proteinases have so far been found only in positive-sense, single-stranded RNA viruses (Gorbalenya and Snijder 1996; Ryan and Flint 1997; Bergmann and James 1999). 3C or 3C-like proteinases are found in all picornaviruses, many related plant viruses and at least two other important families of animal viruses. As these enzymes are distinct from cellular enzymes and their function is essential for viral replication, the 3C proteinases constitute an obvious target for the design of anti-viral drugs (Kräusslich and Wimmer 1988).

Some positive-sense, single-stranded RNA viruses also carry genes coding for chymotrypsin-like serine proteinases or papain-like cysteine proteinases (Kräusslich and Wimmer 1988; Palmenberg 1990; Dougherty and Semler 1993; Ryan and Flint 1997). For other proteinases from these viruses, the enzyme classes to which they belong are not yet established. It is very likely that there are more novel classes of proteinases awaiting discovery in the positive-sense, single-stranded RNA viruses.

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Table 1. The picornaviruses

Genus	Number of serotypes	Viruses	Diseases caused in humans
Enterovirus	>90	Polio virus, coxsackievirus, echovirus	Intestinal infections, poliomyelitis, myocarditis, meningitis, encephalitis, hand, foot and mouth disease, herpangina, myalgia, pleurodynia
Rhinovirus	>100	Rhinovirus	Common cold
Aphthovirus	7	FMDV, equine rhinovirus	Foot and mouth disease of cloven-hoofed animals
Cardiovirus	2	Encephalomyocarditis virus, Theiler's murine encephalitis virus	None known
Hepatovirus	1	Hepatitis A virus	Infectious hepatitis
Parechovirus	2 (3?)	Parechovirus 1, parechovirus 2, Ljungan river virus (?)	Myocarditis, intestinal infections

FMDV, foot and mouth disease virus.

B. Picornaviridae

Picornaviruses constitute a very large family of positive-sense, single-stranded RNA viruses (Rueckert 1996), and some are among the oldest known and best-studied viruses (Landsteiner and Popper 1909; Loeffler and Frosch 1964). Picornaviruses cause a wide variety of different diseases. Presently, viruses of the family Picornaviridae are classified into six genera (Table 1).

The more than 100 serotypes of human rhinoviruses (HRVs) are responsible for most common colds in humans (Couch 1996; Makela et al. 1998). Foot-and-mouth disease virus, the prototype of the aphthoviruses, is the causative agent of one of the most important diseases of livestock (Belsham 1993).

Hepatitis A virus (HAV) is the only known member of the genus *Hepatovirus*, and causes an acute form of infectious hepatitis (Hollinger and Ticehurst 1996). Hepatitis A is still fairly widespread in those parts of the world that do not have safe drinking water supplies. Isolated cases or mini-epidemics of hepatitis A still occur regularly in the developed world and are usually attributed to contaminated food (Pebody et al. 1998). Safe and effective vaccines against hepatitis A have recently become available (Thiel 1998), but their widespread use appears unlikely. Whilst acute HAV infections are, in most cases, relatively harmless, co-infection of patients with chronic hepatitis is often more dangerous (Sjogren 1998; Vento et al. 1998). An increase in

the number of chronic hepatitis infections may therefore change the significance of hepatitis A as an infectious disease.

The genus *Enterovirus* consists of the polio-, coxsackie- and echoviruses (Melnick 1996). These viruses cause a wide variety of illnesses in humans, ranging from mild respiratory tract and intestinal infections to meningitis, myocarditis, encephalitis and poliomyelitis (Table 1). Poliovirus (PV), the major cause of poliomyelitis in humans, has been targeted for world-wide eradication by the turn of the millennium (Cochi et al. 1997; Center for Disease Control 1998). In spite of the success of polio vaccination, it is not clear whether this goal can be achieved (Sutters and Cochi 1997; Taylor et al. 1997). Non-vaccine related cases of poliomyelitis are very rare in most parts of the world.

Enteroviruses remain a serious health problem. A recent epidemic in Asia provided a grim reminder of this (Chang et al. 1998). In many clinical settings, the majority of cases of viral meningitis and myocarditis are caused by enteroviruses.The enteroviruses have also been implicated as triggers of autoimmune diseases such as multiple sclerosis, myocarditis and diabetes (Andreoletti et al. 1997; Carthy et al. 1997; Steinmann and Conlon 1997; Niklasson et al. 1998; Roivainen et al. 1998). Definite proof of a link between enteroviral infections and the onset of autoimmune diseases is still not established. Recently, an animal model of a demyelinating disease that resembles multiple sclerosis and is caused by the picornavirus Theiler's murine encephalitis virus, has provided evidence for a mechanism whereby viruses can trigger autoimmune diseases (Miller et al. 1997).

Two of the echoviruses (EV22 and EV23) have recently been reclassified into a new genus, the parechoviruses. The establishment of the new genus was based partly on observed differences in the mechanism of the proteolytic processing of the polyprotein (Schultheiss et al. 1995).

Theiler's murine encephalitis virus is a member of the genus *Cardiovirus*. It causes the above-mentioned demyelinating disease in mice and constitutes an important model system for these diseases. None of the cardioviruses has been linked to any known disease in humans.

It is very likely that the taxonomy of the Picornaviridae will be further modified in the future. As has happened with the parechoviruses, elucidation of details of the viral replication mechanism may lead to reclassification of individual viruses or the establishment of new genera.

C. Other Families of Positive-Sense, Single-Stranded RNA Viruses

I. Caliciviridae

The caliciviruses were discovered relatively recently and were initially considered to be picornaviruses. Elucidation of details of their structure and repli-

cation mechanism made it clear that they constitute a different family of viruses (Clarke and Lambden 1997; Kapikian et al. 1996). They derive their name (calix is Latin for cup) from cup-like indentations of their capsid that are visible in the electron microscope. Their genome structure is also different from that of the picornaviruses, e.g., the structural proteins are found at the carboxy-terminus of the viral polyprotein.

Several caliciviruses cause intestinal infections in humans. They are now considered to be one of the leading causes of what is often described as a "stomach flu" in humans (Green 1997). The proteolytic processing enzyme of the caliciviruses is a chymotrypsin-like cysteine proteinase (Wirblich et al. 1995; Martín-Alonso et al. 1996).

II. Coronaviridae

The largest of the positive-sense, single-stranded RNA viruses are the coronaviruses. They are enveloped viruses and derive their name from their star-like appearance in the electron microscope. Coronaviruses have developed a more complex replication mechanism than other positive-sense, single-stranded RNA viruses, including several mRNA species (Holmes and Lai 1996). The gene products from the major RNA species are nevertheless produced by specific proteolytic cleavage of the translated polyprotein. The viral proteinase that is responsible for most cleavages is a chymotrypsin-like cysteine proteinase (Tibbles et al. 1996; Liu et al. 1997; Seybert et al. 1997; Lu et al. 1998; Schiller et al. 1998). The 3C-like proteinase of the coronavirus avian-infectious-bronchitis virus cleaves following a glutamine residue, similar to the 3C proteinase of the picornaviruses (Ng and Liu 1998).

Coronaviruses cause upper respiratory tract and intestinal infections in humans and animals and are considered the second major cause of the common cold in humans (Makela et al. 1998). They have also been implicated as a cause of viral diarrhea (Gonzalez et al. 1997).

III. Others

All known positive-sense, single-stranded RNA viruses utilize the strategy of specific proteolytic processing of polyproteins to express their genomes. Some have developed additional strategies, such as subgenomic mRNAs or multiple open reading frames. The specific proteolytic processing of polyproteins by a viral proteinase remains an important part of their replication strategy. Therefore, all known positive-sense, single-stranded RNA viruses carry at least one and often several genes that code for proteolytic enzymes (Kräusslich and Wimmer 1988; Dougherty and Semler 1993).

At least three different classes of proteinases are found in these viruses, and there are probably others awaiting discovery. The chymotrypsin-like cysteine proteinases are so far uniquely found in the three families of viruses discussed above and in related plant viruses. The Nsp4 proteinase of the arteriviruses is a serine proteinase. Analysis of the enzyme and its sequence

led SNIJDER et al. (1996) to propose that the enzyme is more closely related to the picornaviral 3C proteinases than to any serine proteinase.

D. Functions of Viral Proteinases in Positive-Sense, Single-Stranded RNA Viruses

I. The Picornaviral Life Cycle

Figure 1 shows a simplified scheme of the life cycle of hepatitis A virus, a typical picornavirus. It serves to illustrate the significance of the functions of the viral proteinases during viral replication (RUECKERT 1996).

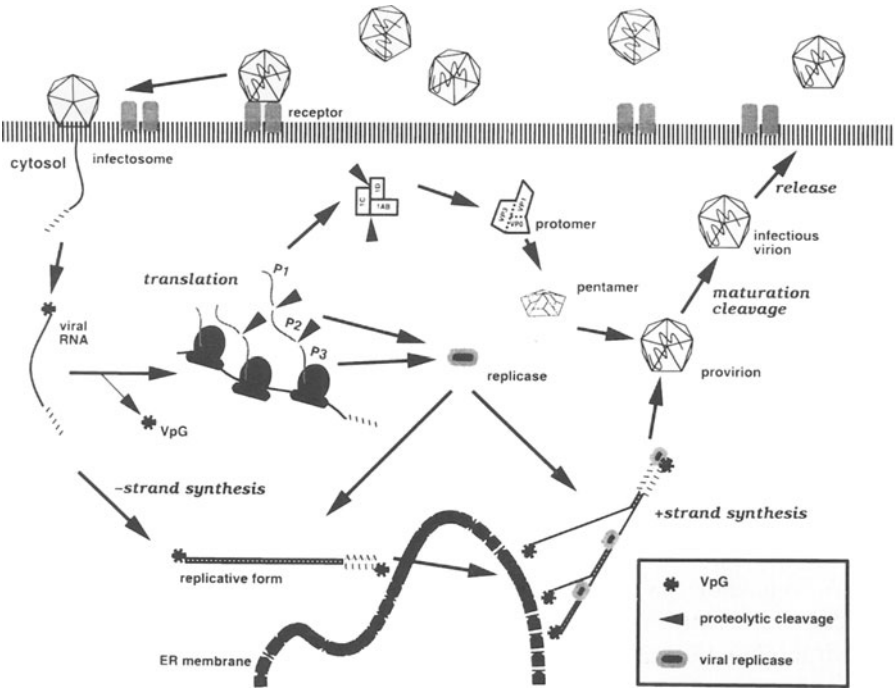

Fig. 1. A simplified scheme of the life-cycle of hepatitis A virus, a typical picornavirus. Viral replication takes place in the cytosol of the host cell. The RNA replication is performed by a viral replicase complex and is localized at modified intracellular membrane structures. Translation of the viral RNA and co-translational, proteolytic processing of the resulting polyprotein is the initial event of a picornaviral infection. The P1 gene products are the structural proteins and are further proteolytically processed to allow capsid assembly. The P2 and P3 gene products are further proteolytically processed and assemble into the viral replicase. In other genera of the Picornaviridae, the P1|P2 cleavage is not performed by the 3C proteinase but by the 2A proteinase or through a completely different mechanism. In the enteroviruses, the cleavages within the structural proteins P1 require the precursor of the 3C proteinase, 3CD

The virus attaches to a specific cell-surface receptor and undergoes some conformational changes that allow it to release its genome into the cytosol of the host cell. A small protein (VPg), which is covalently attached to the 5′ terminus of the picornaviral RNA genome, is cleaved by a host factor, and the resulting RNA is translated into a large polyprotein. The viral polyprotein is co-translationally processed by one or several specific viral proteinases (Palmenberg 1990). The first cleavage typically separates the structural from the non-structural proteins of the virus (Ryan and Flint 1997). The non-structural proteins of the picornaviruses are further proteolytically processed and assemble to form the viral replicase.

Viral replicase complexes perform both negative-sense and positive-sense RNA replication (Porter 1993; Wimmer et al. 1993). The exact composition of the complexes is not clear in even the best-studied picornaviruses (Harris et al. 1994; Xiang et al. 1998). The viral RNA polymerase (the 3D gene product in picornaviruses), the putative RNA helicase (the 2C gene product) and the 3C proteinase form part of this complex. There is also good evidence that some cellular proteins are recruited to form part of the picornaviral-replication complex (Andino et al. 1993; Xiang et al. 1995; Gamarnik and Andino 1997; Parsley et al. 1997; Roehl et al. 1997). Viral replication takes place on modified intracellular-membrane structures. Modification of the intracellular-membrane structures is a common feature of picornaviral infection and is mediated, at least in part, by the 2B and 2C gene products (Bienz et al. 1983; Bienz et al. 1990; Teterina et al. 1997a,b).

The 3C proteinase also has an RNA-binding site and plays a part in the binding of the RNA during the initiation of RNA replication. Small RNA viruses are under evolutionary pressure to maintain the small size of their genome and a limited number of genes. Therefore, many of their gene products, e.g., 3C, have multiple functions. The exact function of the 3C proteinase within the picornaviral replicase complex is not clear. It is possible that some proteolytic cleavages are performed within the replicase complex. For example, the proteolytic cleavage between 3A and 3B could be performed within the replicase complex. 3A is a hydrophobic protein which presumably serves to anchor the replicase complex to modified, intracellular-membrane structures. 3B is the small protein, VPg, which remains covalently attached to the viral RNA (Wimmer 1982).

In a typical picornavirus infection, the ratio of positive-sense to negative-sense RNA is about 50 to 1. Most of the negative-sense RNA exists in the form of a double-stranded replicative form. The VPg-associated positive-sense RNA genome is packaged into provirions, which are transformed into infectious virions by a non-enzymatic proteolytic cleavage of one of the capsid proteins. This is referred to as the maturation cleavage (Palmenberg 1990).

After the structural proteins have been cleaved from the viral polyproteins, their assembly into procapsids is regulated by successive proteolytic cleavages. Only after the proteolytic cleavages have been performed by the

3C proteinase can the capsid proteins undergo the conformational changes that will allow them to assemble into the precursors of the procapsid. Final assembly into the provirion requires the presence of the VPg-associated RNA, but the details of this process are not clear (Rueckert 1996).

There are three distinct functions of viral proteinases during the life cycle of the picornaviruses (Kräusslich and Wimmer 1988). The first is the specific, co-translational, proteolytic processing of the viral polyprotein. The second is the processing of the precursors of the viral capsid. The proteolytic cleavages of the capsid precursor regulate the assembly of the procapsid and should therefore be considered a distinct function of the viral proteinases. A third function of viral proteinases, at least in some picornaviruses, is the cleavage of some host cell proteins (Haghighat et al. 1996; Roehl et al. 1997; Ryan and Flint 1997 and references therein; Yalamanchili et al. 1997). This serves either to downregulate cellular processes that compete with the viral replication or to recruit cellular proteins to become part of the viral-replication machinery.

II. Proteolytic Processing of the Viral Polyprotein

The full-length polyprotein is not detectable under normal conditions because it is already co-translationally processed. The proteolytic cleavages are performed by one or two specific viral proteinases that are themselves part of the polyprotein. The polyprotein processing is performed sequentially. Some of the cleavage sites are cleaved faster than others. Usually, the P1|P2 cleavage, which separates the structural and nonstructural proteins, is the first cleavage event. The mechanism of this primary P1|P2 cleavage is different in the different genera of the picornaviruses (Ryan and Flint 1997).

In the viruses that belong to the genera *Entero-* and *Rhinovirus*, the primary cleavage is performed by a separate 2A proteinase. The 2A proteinase is also a chymotrypsin-like cysteine proteinase and cleaves at its own amino-terminus (Ryan and Flint 1997 and references therein). In aphtho- and cardioviruses, the primary cleavage occurs at the carboxy-terminus of 2A by a non-enzymatic mechanism (Palmenberg et al. 1992; Donnelly et al. 1997). In HAV and, presumably, also in the parechoviruses, the primary cleavage is a 3C-mediated proteolytic cleavage at the amino-terminus of the 2B gene product (Jia et al. 1993; Schultheiss et al. 1994; Martin et al. 1995; Schultheiss et al. 1995).

In all picornaviruses, the majority of the proteolytic cleavages within the polyprotein are performed by the 3C proteinase. The 3C proteinases cleave specifically following a glutamine residue. Additional residues around the scissile bond contribute to the recognition of the cleavage sites (Nicklin et al. 1988; Long et al. 1989; Pallai et al. 1989; Cordingley et al. 1990; Weidner and Dunn 1991; Jewell et al. 1992). These are 4 to 5 residues that precede the scissile bond and 2 to 3 residues that follow it [P_5–P_3' in the nomenclature of Schechter and Berger (1967)].

The 3C-like proteinase of the coronaviruses also specifically cleaves following a glutamine residue (Ng and Liu 1998). The corresponding enzyme in the calicivirus rabbit-hemorrhagic-disease virus specifically recognizes a glutamate residue in the P_1 position of a substrate (Wirblich et al. 1995; Martín-Alonso et al. 1996). The specificities of the 3C and 3C-like proteinases of positive-sense, single-stranded RNA viruses are unique and distinct from those of known mammalian proteinases.

III. Regulation of Capsid Assembly by Proteolytic Cleavages of the Capsid-Protein Precursors

Viral-structural proteins are designed to assemble into large symmetrical structures. They are usually synthesized as precursors to prevent premature assembly or aggregation. The precursor is subsequently covalently modified. The most common form of covalent modification of the capsid precursor in viruses is proteolytic processing (Kräusslich and Wimmer 1988). This is one reason that proteolytic enzymes are common gene products of viruses, even in large DNA viruses.

Two successive proteolytic cleavages by the 3C proteinase are required in picornaviruses to allow capsid assembly (Fig. 1; Rueckert 1996). In the enteroviruses, these cleavages are performed by the precursor 3CD (Ypma-Wong et al. 1988). The RNA-polymerase domain has an effect on the substrate specificity or on the catalytic efficiency, but in the absence of structural information for 3CD it is not clear how this is accomplished. Following the proteolytic cleavages between VP0|VP3 and VP3|VP1, the capsid precursors undergo a conformational change and assemble into pentameric structures. The final assembly of the provirion requires the presence of the RNA (Rueckert 1996).

IV. Inhibition of Cellular Functions by Proteolytic Cleavages of Host Cell Proteins

The third function of proteolytic enzymes in Picornaviruses is to cleave specific cellular proteins. The best-studied example is the cleavage of eIF4G by the 2A proteinase of entero- and rhinoviruses or by the L proteinase of the aphthoviruses (Ryan and Flint 1997). This cleavage impairs translation of capped, cellular mRNAs and therefore improves the translational efficiency of the viral RNA. Not all picornaviruses inhibit host cell translation by this mechanism. Hepatitis A virus carries only a single gene coding for a proteolytic enzyme, 3C (Schultheiss et al. 1994). HAV has no equivalent to either the enteroviral 2A proteinase or the aphthoviral L proteinase. In HAV-infected cells, there is no evidence of a cleavage of eIF4G, and HAV translation even depends on the intact cellular eIF4G. Cleavage of eIF4G by an enteroviral 2A proteinase inhibits HAV replication (Borman and Kean 1997).

There are reports of other cellular proteins that are cleaved by the picornaviral 3C proteinase (ROEHL et al. 1997; RYAN and FLINT 1997 and references therein; YALAMANCHILI et al. 1997). The function of these cleavages in vivo is not completely clear. Some cleavages appear to impair host cell transcription; others may modify cellular proteins to become part of the viral-replication machinery (ROEHL et al. 1997).

E. The 3C Proteinase

I. Structure

Refined crystal structures of the 3C proteinases from three of the six genera of the Picornaviruses have been published (BERGMANN et al. 1997; MATTHEWS et al. 1994; MOSIMANN et al. 1997). Figure 2 (s. appendix, page 400/401) shows a ribbon representation of the three-dimensional structures of the 3C proteinase from HAV and PV. The two enzymes represent two different classes of the 3C proteinases (GORBALENYA and SNIJDER 1996). The HAV 3C proteinase is larger (219 residues); the enzymes from enteroviruses represent a smaller type (183 residues).

The fold of the two-domain structure of the 3C proteinases is similar to that of the chymotrypsin-like serine proteinases (ALLAIRE et al. 1994). The structure consists of two β-barrel domains with identical topology. The proteolytic active site is in a cleft between the two domains, and residues from both domains contribute to the catalytic mechanism and substrate binding (PERONA and CRAIK 1995). There are two alternative descriptions for the structure of the β-barrel domains. They can be described either as a six-stranded β-barrel or a barrel formed by two orthogonal, four-stranded β-sheets in which the edge strands are a part of both sheets (CHOTIA 1984). We feel that the latter description is more appropriate for the larger HAV 3C proteinase (Fig. 2a; BERGMANN et al. 1997). The second and fifth β-strands of each domain are interrupted by a β-bulge or, in the case of β-strand eI, by a single turn of a helix. This introduces a bend into the edge strands (bI, eI, bII and eII) that allows them to continue from one β-sheet to the other. In the smaller enteroviral enzyme, the edge strands are less bent and more continuous (Fig. 2b; MOSIMANN et al. 1997). It is simply the intrinsic twist of the edge β-strands which allows them to wrap around the whole barrel. Each domain of the smaller enteroviral enzyme can be adequately described as a six-stranded β-barrel.

In spite of the differences, the 3C proteinases from HAV and PV show remarkable conservation of their structures. The core of the β-barrel domains superimpose well. The diameter of the two β-barrels, their relative orientation and the direction of the individual β-strands are very similar. A structural superposition of the two structures reveals 30 identical residues in the sequence. The differences between the two 3C proteinases manifest primarily

in the turns and loops which connect the β-strands and in the lengths of the individual secondary-structure elements.

The residues of both the amino- and carboxy-termini of the 3C proteinases form helices. An amino-terminal α-helix is a unique feature of the 3C proteinase. The amino-terminal helix of the 3C proteinase packs against the surface of one of the β-sheets of the carboxy-terminal β-barrel, and the carboxy-terminal helix packs against the amino-terminal domain (Figs. 2, 3, s. appendix, page 400/401). The two helices act like latches in stabilizing the structure.

The proteolytic active site of the 3C proteinase is less accessible than the active site of most chymotrypsin-like serine proteinases. This is primarily due to another structural feature that distinguishes the 3C proteinases from the mammalian chymotrypsin-like enzymes: two β-strands from the carboxy-terminal domain extend past the β-barrel and twist back toward the active site. They form an isolated β-ribbon, with hydrogen bonds formed only between the two β-strands (colored in *light gray* in Figs. 2, 3). This extension of β-strands bII and cII is quite long in HAV 3C, and contributes residues to the active site (BERGMANN et al. 1997); it is nine residues shorter in PV 3C (MOSIMANN et al. 1997).

The molecular surface of 3C on the side opposite from the proteolytic active site is formed by the part of the polypeptide chain that connects the two domains (Fig. 3). The domain connection is flanked by the amino- and carboxy-terminal helices. This region of 3C is important for a function of 3C that is distinct from its proteolytic activity (ANDINO et al. 1990; HÄMMERLE et al. 1992; ANDINO et al. 1993; LEONG et al. 1993; KUSOV and GAUSS-MÜLLER 1997).

II. Specificity and Substrate Binding

Chymotrypsin-like serine proteinases bind their cognate substrates and protein inhibitors in a canonical conformation (READ and JAMES 1986; BODE and HUBER 1992). The proteinases typically bind four to five residues preceding the scissile peptide bond and two to three residues following the site of cleavage [P_5 to P_1 and P_1' to P_3' in the nomenclature of SCHECHTER and BERGER (1967)]. Most of the residues of the peptide substrate adopt a β-strand conformation. The P_1 residue adopts a conformation that represents a tight 3_{10} helix. This causes the carbonyl of the scissile peptide bond to point into the so-called oxyanion hole of the enzyme. The two neighboring substrate residues, P_2 and P_1', adopt a more twisted β-strand conformation to accommodate this. Binding of the peptide substrate involves main-chain hydrogen-bond interactions between the substrate and β-strands of the enzyme, which resembles an anti-parallel β-sheet. As a result of the substrate conformation, side chains of the peptide substrate point into specificity pockets on the surface of the enzyme (PERONA and CRAIK 1995).

There is now evidence, from cocrystal structures of 3C proteinases with bound, peptide-mimetic inhibitors, that peptide substrates bind to the 3C pro-

teinases in a similar mode (DRAGOVICH et al. 1998a, b; WEBBER et al. 1998; BERGMANN and JAMES, unpublished observations). Before these cocrystal structures became available, models were built of the enzyme-substrate interactions (BERGMANN et al. 1997; MATTHEWS et al. 1994; MOSIMANN et al. 1997; WEBBER et al. 1998). The models built utilized the same conformation of the bound substrate and could successfully rationalize the specificity of the 3C proteinases.

The residues from P_5 to P_2 of a substrate form anti-parallel β-sheet interactions with β-strand eII of the proteinase. This interaction is a common feature of enzyme-substrate interactions in chymotrypsin-like proteinases (PERONA and CRAIK 1995). The β-strand b2II in HAV 3C, part of the unique anti-parallel β-ribbon, could form an additional, parallel β-sheet interaction with the P_4 through P_2 residues of a substrate.

The amino-terminal domain of a chymotrypsin-like proteinase provides the majority of the interactions with the substrate residues following the scissile peptide bond (PERONA and CRAIK 1995). In HAV 3C, the P_1' and P_2' residues would interact with the edge of β-strand bI. This β-strand is interrupted by a β-bulge that causes several carbonyl groups of the peptide bonds of this strand to point into the active site. Presumably, they can act as hydrogen-bond acceptors for the binding of a substrate. The β-strand bI of the entero- and rhinoviral 3C proteinases is continuous (Fig. 2b), and there are fewer possible interactions with a substrate in these enzymes. Rhino- and enteroviral 3C proteinases prefer a glycine as the P_1' residue of a substrate (NICKLIN et al. 1988; LONG et al. 1989; PALLAI et al. 1989; CORDINGLEY et al. 1990; WEIDNER and DUNN 1991). It has been suggested that the main chain of the substrate of these enzymes turns at the P_1' residue (MATTHEWS et al. 1994). There is no significant sequence preference for the P_1' residue of a substrate of HAV 3C. We believe that the difference in the conformations of β-strand bI between the two different classes of enzymes results in different conformations of the P_n' residues of the bound substrates.

The cleavage sites for the picornaviral 3C proteinases within the polyprotein are distinguished by the residues in the P_4, P_2, P_1 and P_1' positions (reviewed by BERGMANN 1998; SKERN 1998). All piconaviral 3C proteinases require a glutamine in the P_1 position of a substrate, but the sequence preferences of the enzymes from different viruses for the other positions are distinct. For example, the sequence preference for the residue in the P_4 position of a substrate is different among the various 3C proteinases. The 3C proteinases from entero- and rhinoviruses prefer a small, hydrophobic residue in the P_4 position of a substrate (NICKLIN et al. 1988; LONG et al. 1989; PALLAI et al. 1989; CORDINGLEY et al. 1990; WEIDNER and DUNN 1991). The HAV 3C proteinase prefers a larger, hydrophobic residue (Leu or Ile) in this position (JEWELL et al. 1992). The model of substrate binding places the side chain of the P_4 residue into a hydrophobic cleft formed by β-strands eII, fII and b2II from the carboxy-terminal domain (BERGMANN et al. 1997). The hydrophobic S_4 binding cleft of the entero- and rhinoviral enzymes is smaller than that of

the HAV 3C proteinase (Matthews et al. 1994; Mosimann et al. 1997; Webber et al. 1998). This is due to the fact that several of the residues which form this hydrophobic cleft are larger in the entero- and rhinoviral enzymes (e.g., PV 3C Leu125 and Phe170 correspond to Ala141 and Val 200 in HAV 3C).

All the models of substrate recognition agree that the glutamine residue in the P_1 position of a substrate probably forms a hydrogen bond between the carbonyl oxygen atom of its side chain and the N^{ε} atom of the imidazole ring of a conserved histidine in the S_1 pocket of the 3C proteinases (Matthews et al. 1994; Bergmann et al. 1997; Mosimann et al. 1997). This role of the conserved histidine (His191 in HAV 3C, His160 in HRV, His161 in PV) had been proposed prior to the elucidation of the first crystal structure (Gorbalenya et al. 1989). In the entero- and rhinoviral enzymes, a threonine residue (Thr142) forms an additional hydrogen bond to the side-chain carbonyl of the P_1 glutamine (Webber et al. 1998). There are no suitable groups on the enzyme that can interact with the amide nitrogen atom of the side chain of the P_1 glutamine in the crystal structures of the 3C proteinases. This correlates well with experimental results that show that inihibitors with *N*-substituted glutamine isosteres are effective inhibitors of the 3C proteinases (Malcolm et al. 1995; Morris et al. 1997; Dragovich et al. 1998b; Webber et al. 1998)

How can the picornaviral 3C proteinases distinguish the invariant glutamine residue in the P_1 position of a substrate from glutamate? A mechanism for this distinction has been suggested based on the details of the structure of the S_1 pocket of the HAV 3C proteinase (Bergmann et al. 1997). One edge of the imidazole side chain of the conserved His191 forms part of the S_1 specificity pocket of HAV 3C (Fig. 4a). The N^{ε} atom of the imidazole ring provides a hydrogen-bond donor to recognize the P_1 glutamine side chain. The other edge of the imidazole ring interacts with two buried water molecules in the core of the carboxy-terminal domain of HAV 3C. The water, in turn, interacts with the side-chain carboxyl group of Glu132. Because the side chain of Glu132 is buried inside the hydrophobic environment of the carboxy-terminal β-barrel domain of HAV 3C, it is very likely uncharged. Deprotonation and charging of the side chain of Glu 132, in its hydrophobic environment, would be energetically unfavorable (Qasim et al. 1995 and references therein). Because the two residues, His 191 and Glu132, interact inside the core of the carboxy-terminal β-barrel domain, protonation of His191 would also be energetically unfavorable, much more so than simply having a protonated, positively-charged histidine residue in this environment (Qasim et al. 1995; Bergmann et al. 1997). These interactions thus ensure that His191 of HAV 3C is neutral. A tyrosine residue (Tyr138 in PV 3C) plays a role similar to that of Glu 132 in HAV 3C in the smaller entero- and rhinoviral 3C proteinases (Mosimann et al. 1997).

The available structural information concerning the 3C proteinases can explain the specific recognition of the proteolytic cleavage sites within the viral polyprotein. It is not possible, with the available structural information, to explain why some cleavage sites within the viral polyprotein are preferably

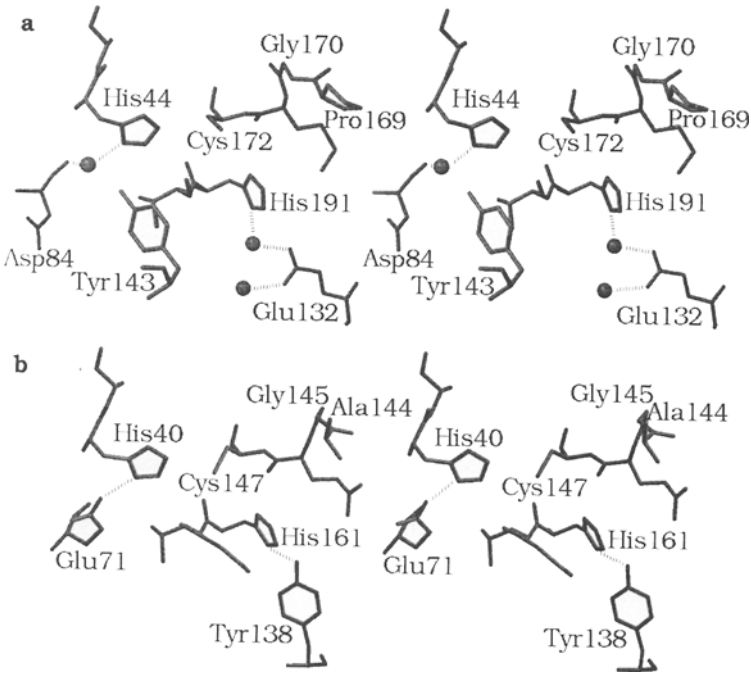

Fig. 4a,b. An all-atom representation of the active sites of the 3C proteinases from (**a**) hepatitis-A virus (HAV) and (**b**) poliovirus in stereo. The views in **a** and **b** are similar. The residues of the oxyanion hole are on the *right*; the residues of the S_1 specificity pocket are *below* it. Water molecules in the structure of HAV 3C are represented by *spheres*

cleaved during the sequential processing of the polyprotein. Presumably, there are other factors that influence the sequence of cleavage events, such as the accessibility or conformation of the cleavage sites.

III. Enzymatic Mechanism

The structure of the active site of the two classes of 3C proteinases is shown in Fig. 4a, b. The three main chemical groups that contribute to the catalytic reaction are in an arrangement which resembles the one in the active site of the chymotrypsin-like serine proteinases (James 1993). The S^{γ} atoms of Cys172 and Cys147 in HAV 3C and PV 3C, respectively, act as the nucleophiles. They are assisted by general acid-base catalysts (His 44 in HAV 3C and His40 in PV) and an electrophilic oxyanion hole. To accommodate the cysteine nucleophile, the active site of the 3C proteinases is larger than that of the chymotrypsin-like serine proteinases. The distance from the S^{γ} atom of the nucleophilic cysteine to the N^{ϵ} atom of the histidine general acid-base catalyst is 3.7–4.0 Å in the various crystal structures of 3C proteinases. This is 0.7–

1.0 Å longer than the analogous distance between the O^{γ} of the nucleophilic serine and the N^{ϵ} atom of the histidine in the serine proteinases.

The oxyanion hole of the chymotrypsin-like proteinases is formed by the NH groups of two peptide bonds, which are in an orientation facilitating donation of hydrogen bonds to the carbonyl of the scissile peptide bond of a substrate (WHITING and PETICOLAS 1994). The conformation of the oxyanion hole is not the lowest-energy conformation. In chymotrypsin-like serine proteinases, the main-chain conformation of the residues which form the oxyanion hole is maintained by interactions of the peptide bonds with other parts of the structure. There are no such interactions in the structures of the 3C proteinases. What, then, maintains the conformation of the oxyanion hole in the 3C proteinases?

Mutation of the nucleophilic cysteine to alanine in the HAV 3C proteinase causes the collapse of the oxyanion hole (ALLAIRE et al. 1994). A similar orientation of the oxyanion hole is observed in the cocrystal structure of a peptide-aldehyde-derived hemithioacetal inhibitor of the HRV 3C proteinase (WEBBER et al. 1998). Apparently, the nucleophile itself has a role in maintaining the conformation of the active site. This provides evidence for the existence of a thiolate-imidazolium ion pair in the active site of the 3C proteinases. The negative charge of the thiolate of the nucleophilic cysteine would assist in orienting the dipole of the peptide bonds of the oxyanion hole. The generally accepted mechanism for other cysteine proteinases assumes a thiolate-imidazolium ion pair in the active site (BROCKLEHURST et al. 1998; STORER and MÉNARD 1994).

Additional groups in the active site of the 3C proteinases, besides the nucleophile, oxyanion hole and general acid-base catalyst, are also important for the enzymatic activity. An aspartate or glutamate residue, corresponding topologically to the third member of the catalytic triad in chymotrypsin-like serine proteinases, is conserved throughout the 3C proteinases (GORBALENYA et al. 1989; GORBALENYA and SNIJDER 1996; RYAN and FLINT 1997). Nevertheless, a true catalytic triad does not exist in these cysteine proteinases (Fig. 4). In the entero- and rhinoviral 3C proteinases, the conserved Glu71 interacts with the imidazole of the histidine general acid-base catalyst in an unusual way (MATTHEWS et al. 1994; MOSIMANN et al. 1997). It forms a hydrogen bond with His 40 through the *anti* lone-pair electrons of its carboxylate (Fig. 4b). This is generally assumed to be a weaker interaction than the more common hydrogen bond through the *syn* lone-pair electrons.

In HAV 3C, the corresponding residue, Asp84, does not interact with the imidazole of His44 at all (BERGMANN et al. 1997; Fig. 4a). A water molecule occupies the position of the carboxylate of a third member of the catalytic triad and is hydrogen bonded to the N^{δ} of His 44. A tyrosine residue interacts with His44. Tyr143 of HAV 3C is perpendicularly above the plane of the imidazole of His44 and, therefore, cannot form a hydrogen bond to the imidazole. Its interaction with His44 must be electrostatic.

The additional groups in the active site of the 3C proteinases, which interact with the histidine general acid-base catalyst, most likely have two func-

tions: they maintain the proper orientation of the active-site residues and they stabilize the charges of those residues. That these interactions appear to be mostly electrostatic and do not form typical hydrogen bonds could be taken as further evidence for an enzymatic mechanism of the 3C proteinases, involving a thiolate-imidazolium ion pair. The details of the actual enzymatic mechanism of the chymotrypsin-like cysteine proteinases remain to be established.

IV. Autocatalytic Excision of the 3C Proteinase

The 3C proteinases can cleave themselves out of the respective viral polyprotein in *cis* when they are part of the polyprotein, or in *trans* when they are expressed separately (Harmon et al. 1992). Palmenberg and Rueckert (1982) were the first to provide evidence that the autocatalytic cleavage of the 3C proteinases could be an intramolecular event. Further evidence was provided by Hanecak et al. (1984). Nevertheless, the available experimental evidence cannot distinguish between a truly intramolecular cleavage and a proteolytic cleavage within a tight dimer or larger oligomer of 3C proteinase precursors.

The three crystal structures of the 3C proteinases suggest a possible model for an intramolecular cleavage event at the amino-terminus of 3C (Matthews et al. 1994; Bergmann et al. 1997; Mosimann et al. 1997). In this model, the unique, amino-terminal α-helix of the 3C proteinases is folded only after cleavage at the amino-terminus of 3C. Prior to the cleavage, only the last turn of helix A exists as a reverse turn, and the amino-terminal residues reach into the active site along β-strand bI in an extended conformation. The last turn of helix A is formed by a conserved sequence motif, R/K-R/K-N-I/L. After the amino-terminus is cleaved, the folding of the stable helix A removes the P_n' residues from the active site to prevent intramolecular product inhibition.

It is very difficult to imagine a similar intramolecular cleavage at the carboxy-terminus of 3C. The authors of all the published crystal structures instead favor an intermolecular proteolytic cleavage within a tight polymer of 3C precursors (Matthews et al. 1994; Bergmann et al. 1997; Mosimann et al. 1997). All three crystal structures are made up of at least two independent molecules. In all the structures, the carboxy-terminus of one molecule of a dimer is within reach of the proteolytic active site of another molecule. However, the dimers in the three different crystal structures are different, and there is no independent experimental evidence for the formation of a tight dimer of the 3C proteinases in solution. Structural work on larger precursors of 3C will be required to resolve the mechanism of autocatalytic cleavage.

V. Other Functions of the Picornaviral 3C Gene Product

The most conserved motif in the sequence of the picornaviral 3C proteinases is not part of the proteolytic active site (Gorbalenya et al. 1989; Ryan and Flint 1997). It was first shown for poliovirus 3C that these residues are important for a function of the 3C gene product which is distinct from the prote-

olytic activity (Andino et al. 1990; Hämmerle et al. 1992; Andino et al. 1993). That the few gene products of small RNA viruses perform multiple functions in viral replication is not an uncommon situation. The picornaviral 3C proteinase is recruited as part of the viral replicase complex and has an RNA-binding site. The RNA-binding activity of 3C is important for the recognition of the non-translated regions of the viral RNA during RNA replication (Andino et al. 1990; Hämmerle et al. 1992; Andino et al. 1993; Leong et al. 1993; Harris et al. 1994; Kusov and Gauss-Müller 1997; Walker et al. 1995). The exact function of 3C during viral RNA replication is not known. It is possible that the RNA-binding activity of 3C simply serves to recruit the proteinase to the replicase complex in order to perform essential proteolytic cleavages within these complexes (Xiang et al. 1998).

The conserved RNA-binding site of 3C is on the surface of the molecule opposite from the proteolytic active site (Matthews et al. 1994; Bergmann et al. 1997; Mosimann et al. 1997). The conserved sequence motif KFRDI forms part of the connection between the two domains of the 3C proteinase (Fig. 3). The domain connection is in a partly helical and partly extended conformation and is flanked by the amino- and carboxy-terminal helices. Several of the β-turns that connect the strands of the two β-barrel domains also contribute to this surface. This surface of the molecule is highly charged (Bergmann et al. 1997).

The RNA-binding site of 3C is on the opposite site of the proteolytic active site. Therefore, the structures suggest that the two activities could be completely independent. Both the amino- and the carboxy-terminal helices do, however, contribute to the RNA-binding site. Therefore, RNA binding could have an influence on the processing of the termini of 3C (Ryan and Flint 1997). Similarly, a larger precursor of 3C, such as 3ABC or 3CD, would most likely have different RNA-binding activity. The RNA-binding activity presents another possible target for the design of antiviral inhibitors. Because little is known about the molecular details of the RNA-binding activity of 3C, there has been little effort directed against this function to date.

F. Inhibition of the 3C Proteinase

I. Effect of 3C Proteinase Inhibitors on Viral Replication

The 3C proteinase performs an important and indispensable function during the viral life cycle. The chymotrypsin-like cysteine proteinases also represent a unique class of proteolytic enzymes, with a specificity that is distinct from all known cellular proteinases (Gorbalenya and Snijder 1996; Ryan and Flint 1997; Bergmann and James 1999). As such, the picornaviral 3C proteinases represent ideal targets for the design of proteinase inhibitors with antiviral activity (Kräusslich and Wimmer 1988). Some 3C proteinase inhibitors effectively inhibit viral replication and reduce viral load when tested in cell cul-

tures (Morris et al. 1997; Dragovich et al. 1998a, b; Kong et al. 1998; Webber et al. 1998).

Even for the best-reported proteinase inhibitors, the ex vivo inhibition of viral replication in cell cultures is usually significantly less potent than the in vitro proteinase inhibition. For very good proteinase inhibitors with nanomolar inhibition constants (K_is), the doses producing a response in 50% of animals are typically micromolar or slightly below. The antiviral activity of some good proteinase inhibitors was disappointing when tested ex vivo; these inhibited viral replication in cell culture not at all or only at concentrations that are toxic for the cells (Webber et al. 1996). Effective antivirals need to be tight-binding proteinase inhibitors, possess low toxicity and be able to reach sufficiently high intracellular levels.

II. Strategies for the Design of 3C Proteinase Inhibitors

It is important to keep in mind that the recent successful development of HIV-proteinase inhibitors has benefited tremendously from the understanding of the mechanism of aspartic proteinases, which was derived from many years of experimental work on other aspartic proteinases, such as renin. The chemical functionality at the core of all the new anti-HIV drugs, which are HIV-proteinase inhibitors, is very similar to classical aspartic proteinase inhibitors (Hoetelmans et al. 1997; Korant and Rizzo 1997; see also the introduction to this volume). This illustrates the point that a detailed understanding of enzymatic structure, function and mechanism is invaluable for the design of effective inhibitors. While there exists a considerable amount of information about specific intermolecular interactions between 3C proteinases and their substrates or inhibitors, little is known about the catalytic mechanisms of these enzymes.

The most commonly applied approach to the development of 3C proteinase inhibitors combines a reactive chemical functionality with groups that satisfy the known specificity determinants of the proteinases. The reactive chemical functionalities are usually groups that are known to react covalently with the active-site thiol nucleophile of cysteine proteinases (Rasnick 1996). It has been shown that several inhibitors react covalently with the active-site thiol of the 3C proteinases. To achieve specificity, these functionalities are combined with groups that mimic the specificity determinants of a peptide substrate of the proteinase. Several sources of experimental information contributed information about the specificity requirements of the 3C proteinases.

Analysis of the sequence of the cleavage sites within the natural substrate, the viral polyprotein, usually reveals sequence preferences (reviewed by Bergmann 1998 and Skern 1998). Kinetic studies with small peptide substrates are also informative in identifying the substrate preferences of the proteinase (Nicklin et al. 1988; Orr et al. 1989; Pallai et al. 1989; Cordingley et al. 1990; Weidner and Dunn 1991; Jewell et al. 1992). Most of these studies found that

the 3C proteinases prefer certain amino acids in the P_4, P_2 and P_1 positions of the substrate. The entero- and rhinoviral enzymes also require glycine and proline in the P_1' and P_2' positions of a substrate. The sequence preferences for substrates of the 3C proteinases from individual viruses differ. It is also noteworthy that the optimal cleavage sequence found in the context of a hexapeptide substrate can be different from the consensus sequence of the cleavage sites in the viral polyprotein. It is generally assumed that the cleavage sequence preferences derived from kinetics studies with small peptide substrates are more useful for the design of specific inhibitors.

The third source which contributes experimental information about the specific interactions between the 3C proteinases and their cognate substrates are the crystal structures of 3C proteinases (MATTHEWS et al. 1994; BERGMANN et al. 1997; MOSIMANN et al. 1997) and, more recently, proteinase-inhibitor complexes (WEBBER et al. 1996; DRAGOVICH et al. 1998 a, b; WEBBER et al. 1998). Initially, the crystal structures of the free enzymes were used to model the binding of a substrate or inhibitor. Crystal structures of inhibitor complexes proved the general validity of the binding modes utilized in these models. There were, however, differences in the details of the enzyme-substrate interactions between the models and the corresponding cocrystal structures (WEBBER et al. 1998). While theoretical models of enzyme-substrate interactions are useful in the absence of experimental structures, they rarely predict all details of intermolecular interactions correctly or as accurately as an actual cocrystal structure of a complex.

The detailed understanding of the specific interactions between the 3C proteinases and their preferred substrates has led to the development of potent inhibitors of the enzymes. Most of these inhibitors mimic the specific interactions of a P_4 to P_1 tetrapeptide substrate and combine this with a chemical functionality which reacts covalently with the active-site thiol (KALDOR et al. 1995; MALCOLM et al. 1995; MORRIS et al. 1997; DRAGOVICH et al. 1998 a,b; KONG et al. 1998; WEBBER et al. 1998). The chemical functionalities utilized are usually classic cysteine proteinase inhibitors that react covalently with the enzyme. In the case of the rhinovirus 3C proteinase, these inhibitors have been further improved by optimization of the individual groups that target the specific subsites of the enzyme (DRAGOVICH et al. 1998a,b; KONG et al. 1998; WEBBER et al. 1998). This has resulted in some potent proteinase inhibitors having sub-nanomolar inhibition constants.

There have also been alternative approaches to the discovery and design of 3C proteinase inhibitors. An interesting method used to identify specific inhibitors of the HAV 3C proteinase has been employed by MCKENDRICK et al. (1998). HAV 3C proteinase was incubated with a mixture of peptide-based covalent inhibitors, and the inhibited enzyme was analyzed by mass spectrometry. The analysis showed that the enzyme was able to select from the mixture one inhibitor that best fit its specific interactions. This approach could be used generally to optimize enzyme inhibitors that reacted covalently. In this

case, it also identified peptide-mimetic inhibitors that targeted the specific S′ subsites of the enzyme.

A very labor-intensive method is the screening of large libraries of natural compounds or cultures of microorganisms for enzyme inhibitors (Singh et al. 1991; Kadam et al. 1994; McCall et al. 1994; Brill et al. 1997; Jungheim et al. 1997). When successful, this approach can identify completely new and unexpected classes of compounds. The resulting compounds are rarely very potent inhibitors; none of the proteinase inhibitors that resulted from these screening procedures have been developed into potent inhibitors.

III. Inhibitors of the Chymotrypsin-Like Cysteine Proteinases

The most effective 3C proteinase inhibitors combine a chemical group that interacts covalently with the active-site cysteine nucleophile with other groups that interact non-covalently with the specificity determinants of the enzyme. The chemical functionalities that react covalently with the nucleophilic thiol of the enzyme are classical cysteine proteinase inhibitors (Rasnick 1996). Among the reactive groups are aldehydes (Kaldor et al. 1995; Malcolm et al. 1995; Shepherd et al. 1996), iodoacetylpeptidyl amides (McKendrick et al. 1998), β-lactams (Skiles et al. 1990), halomethyl ketones (Orr et al. 1989; Sham et al. 1995; Morris et al. 1997), isatins (Webber et al. 1996) and vinylogous sulfones and esters. The best, presently known, inhibitors of the 3C proteinases are the vinylogous esters (Dragovich et al. 1998 a,b; Kong et al. 1998). These compounds react with the nucleophilic thiol of the enzyme via a Michael addition. This was confirmed by experimental evidence, including cocrystal structures of proteinase-inhibitor complexes (Dragovich et al. 1998a,b). Other chemical functionalities also provide potent proteinase inhibitors but are far less promising as antivirals. No results of experiments regarding the toxicity and bioavailability of 3C proteinase inhibitors in animals or humans have been published at this time.

The most commonly encountered problems, when proteinase inhibitors were tested for their antiviral activity in cell cultures, were toxicity and poor intracellular availability. Some very promising proteinase inhibitors, such as the isatins, showed no antiviral efficacy at concentrations below their toxicity levels in cell culture (Webber et al. 1998). Presumably, problems with toxicity are, at least in part, due to the reactivity of the covalent 3C proteinase inhibitors, but no potent, non-covalent inhibitors of the 3C proteinases are known at this point. Other potent proteinase inhibitors are significantly less effective as antivirals in cell cultures. Among those inhibitors are the fluoromethyl ketones and vinylogous sulfones. Presumably, these inhibitors do not achieve sufficiently high intracellular concentrations.

The minimum size of the effective 3C proteinase inhibitors corresponds to the equivalent of a tetrapeptide which mimics the P_4 to P_1 residues of a substrate. Smaller inhibitors are significantly less effective because of the reduced

number of specific, intermolecular interactions they can form with the enzyme. The problem of poor intracellular availability, which is encountered with some proteinase inhibitors, can therefore not be overcome by reducing the size of the inhibitors.

G. Summary and Outlook

The 3C proteinases perform an essential function during the life cycle of three large families of animal viruses (Picornaviridae, Caliciviridae and Coronaviridae). Members of these virus families are responsible for a large number of respiratory and intestinal infections and also cause more serious viral infections. The chymotrypsin-like cysteine proteinases of positive-sense, single-stranded RNA viruses constitute a distinct class of enzymes, and the specificity of the 3C proteinases is unique. They are, therefore, an ideal target for the design of specific antiviral drugs. Extensive kinetic studies and crystal structures of the enzymes from three genera of the Picornaviridae have provided important insights into the structure–function relationships of these enzymes, but little is known about the enzymatic mechanism of the 3C proteinases. So far, all effective inhibitors of the 3C proteinase react covalently with the active-site cysteine nucleophile.

Rhinoviruses and some of the coronaviruses are together responsible for the vast majority of common colds. Both could potentially be inhibited by effective 3C proteinase inhibitors. Because many viruses from different families cause upper-respiratory-tract infections, which are essentially indistinguishable by their clinical symptoms alone, effective treatment would also require simple analytical procedures to identify the causative agent. Members of the family Caliciviridae and Coronaviridae cause a large number of intestinal infections, often referred to as "stomach flu". These viruses should, therefore, be considered attractive drug-design targets. A treatment for the rarer but often serious enteroviral infections would be of great value. Whether or not a treatment of enteroviral infection could be beneficial for the prevention of autoimmune diseases is not clear.

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