Chapter 6
Picornaviruses

David Neubauer, Jutta Steinberger, and Tim Skern

Abstract The picornavirus family contains several major human and animal pathogens. Vaccines against some of these pathogens are available. However, the availability of potent antiviral compounds would be an appreciable advantage in fighting these pathogens. Inside their non-enveloped capsid, picornaviruses possess a positive sense RNA genome with a single open reading frame. Upon release into the cytoplasm, the genome is translated into a single polyprotein that is processed by virally encoded proteinases. These proteinases represent excellent targets for the development of anti-virals for two reasons. First, efficient polyprotein processing is essential for successful viral replication. Second, the picornaviral proteinases show notable differences to cellular proteinases. To aid in the development of anti-virals, detailed knowledge of the mechanisms, substrate specificities and structures of these proteinases is needed. This chapter reviews recent progress, discusses selected substances with antiviral activity against picornavirus proteinases and outlines several new avenues for the design of novel anti-virals.

Keywords Poliovirus • human rhinovirus • aphthovirus • proteolytic processing • translational control

6.1 Introduction

The family of the positive-strand picornaviruses includes a number of important human and animal pathogens such as poliovirus (PV), hepatitis A virus (HAV), coxsackievirus (CV), human rhinovirus (HRV) and foot-and-mouth disease virus (FMDV) (Racaniello, 2007). Of these pathogens, only PV, HAV and FMDV can be controlled by vaccination. There is thus a clear need for anti-viral agents to combat HRV and CV. In addition, although the vaccines against FMDV and PV have proven effective in most cases, they are not perfect. For instance, the FMDV

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U. Lendeckel and N.M. Hooper (eds.), *Viral Proteases and Antiviral Protease Inhibitor Therapy*, Proteases in Biology and Disease 8,

vaccine can be only used under certain circumstances proscribed by regulatory authorities such as the European Union. Furthermore, the present PV vaccines may not be sufficient to finally achieve and maintain the eradication of PV and may need support from specific anti-viral agents (Aylward et al., 2005). Thus, in addition to the need for anti-virals against HRV and CV, there is also a need for anti-viral compounds against PV and FMDV.

The last 25 years have seen an enormous increase in our knowledge and understanding of the molecular biology and pathogenicity of many family members (see Semler and Wimmer, 2002) and a large number of substances have been shown to possess activity against picornaviruses and/or the proteins that they encode (reviewed in De Palma et al., 2008). Nevertheless, at present no anti-viral substances have been approved for clinical use against picornaviral infections.

This chapter begins by explaining the situations in which an anti-viral against a particular picornavirus would be advantageous, identifies the possible proteolytic activities against which anti-viral substances can be directed as well as reviewing past progress and future directions.

6.1.1 Poliovirus

PV is the subject of a WHO eradication program, initiated in 1988 and originally targeted for completion in 2000 (Robertson et al., 1990). However, the number of cases of wild-type PV reported worldwide in 2007 was 1,310; in the first 5 months of 2008, reports of 354 cases had been received (Anon, 2008). Several reasons for the lack of success in completely eradicating PV are apparent. There is the often low level of immunity induced by the trivalent oral vaccine, a failure to immunise all children, sometimes for religious reasons, and the emergence of recombinant, virulent strains of PV derived from the vaccine strains themselves (Andrianarivelo et al., 1999; Katz, 2006). Furthermore, wild-type strains of PV are still being detected in parts of Africa, even though cases of disease have not been reported (E. Wimmer, personal communication). Finally, certain immune-suppressed patients are capable of shedding PV for many years without showing symptoms (MacLennan et al., 2004; Yoneyama et al., 2001).

Several of the above problems indicate that both wild-type and vaccine-derived PV strains will still be circulating when the WHO eventually recommends the cessation of vaccination against PV. The non-vaccinated population, which would grow every year, would therefore be at risk for infection from any circulating PV. To combat this eventuality, the WHO has made a series of recommendations (Aylward et al., 2005). One recommendation for treating post-vaccination outbreaks of poliomyelitis is the use of an anti-PV agent (Aylward et al., 2005). The suitability and feasibility of anti-viral agents directed against PV was discussed at a meeting convened by the National Academy of Sciences (NAS) of the USA in November 2005. Subsequently, the NAS recommended that such anti-viral agents should be developed to treat post-vaccination outbreaks

and to treat immune-comprised patients suffering from persistent infections (N.R.C. Committee on Development of a Polio Antiviral and Its Potential Role in Global Poliomyelitis Eradication, 2006). Further arguments for the development of anti-viral agents against poliovirus can be found in a recent review (Collett et al., 2008).

6.1.2 Coxsackievirus

Coxsackieviruses, like PV, also belong to the enterovirus genus of the picornavirus family. Coxsackie B viruses have long been recognised as one of the most significant causes of dilated cardiomyopathy, a major contributor to fatal heart failure in the developed world (Baboonian et al., 1997). One pathway to cell damage during infection is the cleavage of the structural protein dystrophin in myocytes by a virally encoded proteinase (Badorff et al., 1999). This loss of dystrophin also aids the production and release of virus particles (Badorff and Knowlton, 2004). The availability of anti-viral agents to prevent this damage during cardiac surgery and convalescence from heart disease would be of great benefit.

6.1.3 Human Rhinoviruses

Human rhinoviruses are the major causative agents of the common cold (Arruda et al., 1997). The replication of these viruses is usually confined to the upper respiratory tract and the illness is usually mild and not dangerous. However, recent investigations have revealed connections to diseases of the lower respiratory tract such as pneumonia and influenza-like disease (Turner, 2007). Furthermore, rhinovirus infections may aggravate the condition of asthma sufferers (Johnston et al., 1993). Two genetic groups of HRVs (HRV-A and HRV-B) have been recognised for some time and found associated with disease (Savolainen et al., 2002). Recently, however, a new genotype of HRV, HRV-C, has been detected in patients suffering from influenza-like disease in which influenza virus could not be detected (McErlean et al., 2007). Viruses from this HRV-C genetic group have now been shown to be causes of hospitalisation in children across the globe (Lau et al., 2007; Renwick et al., 2007). Investigations into the properties of this newly discovered HRV genotype have not yet begun, as it has not been possible to propagate the viruses in cell culture. However, comparison of the available full length genomic sequences shows clearly that these viruses represent a new genetic group. Given the sheer numbers of HRV serotypes, control by vaccination is not an option. However, the availability of inhibitors to treat asthma patients and children hospitalised with respiratory disease brought about by HRV infections would be of significant value in the clinic. This topic has been recently reviewed by Patick (Patick, 2006).

6.1.4 Foot-and-Mouth Disease Virus

Foot-and-mouth disease virus (FMDV), a veterinary pathogen of major economic importance, is still endemic in Africa, Asia and parts of South America (Thomson et al., 2003). The disease is controlled by vaccination and by bans on the movement of infected animals as well as the export of products from infected animals (Grubman and Baxt, 2004). For endemic areas, an anti-viral would not be useful for the simple reasons of cost and the possibility of resistance. There are however two scenarios in which an anti-viral agent would be useful for the treatment or prevention of FMDV infections. These are in the treatment of outbreaks in areas which have been free of FMDV (e.g. in England in 2007) and in the treatment of persistent infections. Persistently infected animals are a problem in Africa, affecting buffaloes and impala (Thomson et al., 2003). The virus excreted by such animals can be transmitted to local farm stock and cause difficulty in the sale and movement of these animals. The availability of anti-viral agents against FMDV would make it possible to test whether persistently infected animals can be cured of the infection.

6.1.5 Hepatitis A virus

Since the 1990s, control of the single serotype HAV has been possible using an inactivated vaccine that induces a strong and protective antibody response. Nevertheless, in 2003, over 7,000 reported cases of HAV were reported in the USA alone, the majority being adults (Brundage and Fitzpatrick, 2006). There is thus still a need for the development of an anti-viral to shorten the period of illness, mitigate its symptoms and reduce the time of convalescence. An anti-viral would be of appreciable importance to those patients who suffer from relapses as well as the 1% of patients suffering liver failure and requiring a liver transplant (Brundage and Fitzpatrick, 2006).

6.1.6 Proteolytic Targets for Anti-viral Compounds in Picornaviruses

The above examples illustrate the need for further measures to control these members of the picornavirus family. In order to discuss the potential targets for anti-viral agents encoded by the picornaviruses, it is necessary to consider how the information present in the picornavirus genome is expressed. Picornaviruses possess a genome of positive polarity which is surrounded by a non-enveloped capsid comprising 60 copies of each of the four capsid proteins, VP1–VP4. The information in the genome is first expressed as a large polyprotein which is cleaved

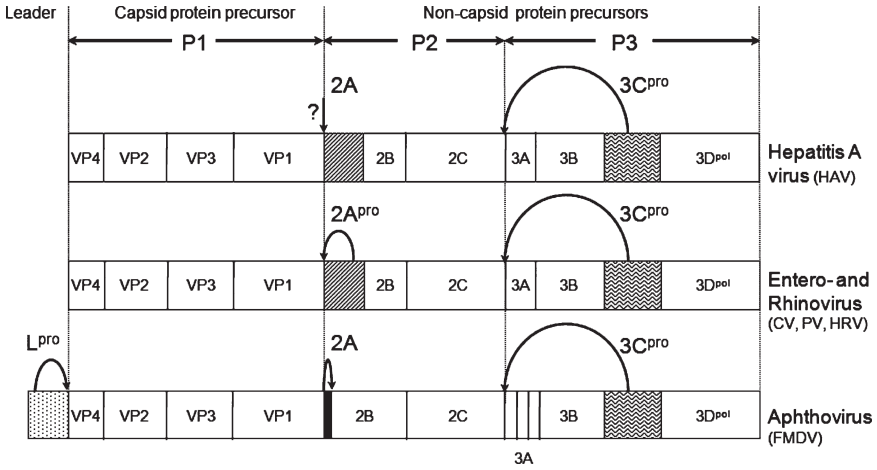

Fig. 6.1 Proteolytic processing in picornaviruses. Variations in primary cleavage events in four picornavirus genera. The polyproteins of the indicated viruses are shown schematically as open boxes. Primary cleavages are indicated. The different shadings of the 2A protein reflect differences in mechanism and size in this protein between the different genera. The proteolytic agent separating VP1 from 2A in HAV is believed to be an as yet unidentified host cell protease (Morace et al., 2008) (Adapted from Skern et al., 2002)

into the mature viral proteins by virally encoded proteinases (Racaniello, 2007; Fig. 6.1). Hepatoviruses (HAV) encode just one proteinase whereas enteroviruses (PV and CV), rhinoviruses and aphthoviruses (FMDV) all encode two proteinases for processing. The 3C protease ($3C^{pro}$), a chymotrypsin-like cysteine proteinase responsible for the majority of cleavage events, is found in all picornaviruses. The second proteinase encoded by entero- and rhinoviruses, the 2A protease ($2A^{pro}$), is also a cysteine proteinase with a chymotrypsin-like fold. In contrast, the second proteinase encoded by FMDV, the Leader protease (L^{pro}), is a papain-like cysteine proteinase (Skern et al., 2002). All three of these proteinases differ in many ways from the proteinases of the host and consequently represent drug targets. The 2A protein of FMDV has, in contrast, no proteolytic activity. Instead, the RNA sequence encoding the last three amino acids of the 2A interrupts the synthesis of the polyprotein chain. This particular sequence on the RNA leads the ribosome to pause and release the structural precursor. However, a certain percentage of the ribosomes remain attached to the RNA and continue translation without changing the reading frame. This allows the non-structural part of the polyprotein to be translated, but at reduced levels compared to the structural precursor. This mechanism is referred to as a ribosome skip (Atkins et al., 2007). A third target for anti-viral agents in all picornaviruses is, of course, the viral RNA polymerase ($3D^{pol}$), the enzyme replicating the viral RNA for which there is no cellular counterpart.

Over the years, most attention has focussed on the $3C^{pro}$ and $3D^{pol}$ enzymes because they are common to all picornaviruses and are involved in many steps of processing and replication. Indeed, a large body of information on the structures

Table 6.1 Overview of known structures of picornaviral proteases

Protease	Virus	Method	Resolution	PDB-entry	Reference
$3C^{pro}$	HRV2	X-Ray	1.85Å	1CQQ	Matthews et al. (1999)
	HRV14	X-Ray	2.30Å	Not available	Matthews et al. (1994)
	HRV14	NMR		2IN2	Bjorndahl et al. (2007)
	PV1	X-Ray	2.10Å	1L1N	Mosimann et al. (1997)
	HAV	X-Ray	2.00Å	1HAV	Bergmann et al. (1997)
	FMDV	X-Ray	1.90Å	2BHG	Birtley et al. (2005)
	CVB3	X-Ray	2.40Å	2VB0	Anand et al., unpublished
$3CD^{pro}$	PV	X-Ray	3.00Å	2IJD	Marcotte et al. (2007)
$2A^{pro}$	HRV2	X-Ray	1.95Å	2HRV	Petersen et al. (1999)
	CVB4	NMR		1Z8R	Baxter et al. (2006)
L^{pro}	FMDV	X-Ray	3.00Å	1QOL	Guarné et al. (1998)
	FMDV	NMR		2JQF	Cencic et al. (2007)
sL^{pro}	FMDV	NMR		2JQG	Cencic et al. (2007)

and biochemistry of these enzymes is available (for $3C^{pro}$ see Table 6.1; for $3D^{pol}$ see Ferrer-Orta et al., 2007; Hansen et al., 1997; Lyle et al., 2002; Marcotte et al., 2007). Furthermore, inhibitors to both enzymes have been developed (Dragovich et al., 1998a; Harki et al., 2006; Huitema et al., 2008) and at least one $3C^{pro}$ inhibitor (AG-7088, also known as rupintrivir) has been tested in the clinic (Hayden et al., 2003; Witherell, 2000). The structures of picornaviral proteinases that have been determined are summarised in Table 6.1.

Experience with anti-viral agents against human immunodeficiency virus (HIV) has shown that it is important to have more than one target for anti-viral agents to combat the development of resistance in viruses with RNA genomes. Nevertheless, in contrast to the situation with $3C^{pro}$ and $3D^{pol}$, interest in the $2A^{pro}$ of entero- and rhinoviruses and the Lb^{pro} of FMDV as drug targets has been limited. This lack of interest is based on the fact that both $2A^{pro}$ and Lb^{pro} perform just one single intramolecular cleavage on their respective polyprotein. Given the rapid kinetics of these reactions (Glaser et al., 2001; Glaser et al., 2003), the general consensus of opinion has been that it will be difficult to inhibit these reactions in the infected cell. However, recent work by Crowder and Kirkegaard (Crowder and Kirkegaard, 2005) has suggested that the inhibition of $2A^{pro}$ cleavage by anti-viral agents would actually be a very effective strategy to block replication of those picornaviruses encoding such an enzyme. Crowder and Kirkegaard showed that mutations in $2A^{pro}$ had a trans dominant effect on the replication of the wild-type virus. Thus, co-transfection of a wild-type PV RNA with a PV RNA containing a debilitating mutation in $2A^{pro}$ led to a reduction in the replication of the wild-type virus. The simplest explanation of this result is that the mutant virus fails to free the $2A^{pro}$ from the capsid protein precursor. This capsid precursor with the $2A^{pro}$ extension can also be incorporated into the assembling wild-type capsid; however, the $2A^{pro}$ extension prevents completion of the capsid and thus has a detrimental effect on the assembly and virus production of the wild-type. This phenomenon has two implications for the targeting of the $2A^{pro}$. First, it is not necessary for the $2A^{pro}$ to

be inhibited completely. A few incorrect capsid precursors will be able to interfere with a large number of correctly processed ones. Second, it will be theoretically more difficult for the virus to develop resistance to the inhibitor because the presence of sensitive viruses will upset the replication of any resistant mutants which may arise (Crowder and Kirkegaard, 2005; Semler, 2005).

A similar situation can be imagined in FMDV with mutants that prevent processing by the Lb^{pro}. As mentioned above, Lb^{pro} also just carries out one intramolecular cleavage on the viral protein; unlike $2A^{pro}$, this cleavage is between its own C-terminus and the N-terminus of the capsid protein precursor (Fig. 6.1). This will eventually lead to the generation of an N-terminal extension of VP4. Incorporation of such molecules into the capsid can be expected to have a detrimental effect on viral infectivity similar to that found with the $2A^{pro}$ extension of VP1 in PV.

In order to make use of the observation of Crowder and Kirkegaard (Crowder and Kirkegaard, 2005), a thorough knowledge of the molecular mechanisms of the $2A^{pro}$ and Lb^{pro} is required. Over the years, a substantial amount of biochemical and structural information on these enzymes has been generated (Table 6.1; reviewed in Skern et al., 2002; Baxter et al., 2006; Cencic et al., 2007). However, much knowledge of fundamental importance, such as how $2A^{pro}$ specificity is defined and whether there is a difference between the intra- and intermolecular cleavage reactions, remains outstanding.

6.2 Picornaviral 3C Proteinases

The first investigations of the protein processing cascade in picornaviruses used inhibitor profiling to identify $3C^{pro}$ as a member of the cysteine protease family (Gorbalenya and Svitkin, 1983; Pelham, 1978). Subsequently, comparison of protein sequences and protein modelling revealed that the $3C^{pro}$, although cysteine proteases, possess a similar fold to that of chymotrypsin (Argos, 1984; Bazan and Fletterick, 1988; Gorbalenya et al., 1989). The catalytic triad of the $3C^{pro}$ was, however, predicted to comprise the residues cysteine, histidine and glutamate (Gorbalenya et al., 1989). The first structures of the $3C^{pro}$ of HAV (Allaire et al., 1994) and HRV14 (Matthews et al., 1994) confirmed these predictions of a chymotrypsin fold. Furthermore, the catalytic triad of the HRV14 $3C^{pro}$ did indeed have glutamate as the third residue of the active site. The situation in the HAV enzyme was however less clear. Initially, James and co-workers favoured the hypothesis that residue tyrosine 143 oriented the active site histidine residue (Allaire et al., 1994; Bergmann et al., 1997). However, in later work using an alternative crystal form, the active site had rearranged to allow aspartate 84 to orient the histidine, as seen in the canonical chymotrypsin-like enzymes (Yin et al., 2005). Subsequent structures of $3C^{pro}$ from other genera revealed that the third member of the catalytic triad was glutamate in PV $3C^{pro}$ and aspartate in the FMDV enzyme (Birtley et al., 2005; Mosimann et al., 1997).

The $3C^{pro}$ are the workhorses of the picornaviral processing reaction. Starting at the 2C/3A junction, they cleave to generate all the mature proteins shown in Fig. 6.1,

except at the site between VP4 and VP2 and at the sites mentioned above for which the L and 2A proteinases are responsible. The VP4/VP2 cleavage takes place during maturation of the viral capsid by an as yet unidentified proteolytic activity.

The sequences at which the $3C^{pro}$ cleave vary between the genera. The most specific enzymes are the PV and HRV $3C^{pro}$, requiring a small residue at P4, glutamine at P1 and glycine at P1′. The HRV $3C^{pro}$ in particular also shows a strong preference for proline and other hydrophobic residues at the P2′ position (Duechler et al., 1987; Skern et al., 2002). In contrast, the HAV $3C^{pro}$ has a preference for a bulky, hydrophobic residue at P4, a small aliphatic residue at P2 and glutamine or glutamate at P1. The HAV $3C^{pro}$ has little or no specificity at the P′ side (Bergmann et al., 1997). Like the HAV enzyme, the FMDV $3C^{pro}$ can also accept glutamine or glutamate at P1 and prefers a bulky residue at P4. The P2 residue is frequently lysine or threonine. At the P1′ position, the enzyme can accept glycine, serine and a variety of large hydrophobic residues (Birtley et al., 2005). The structural basis for these cleavage requirements are briefly explained for the individual enzymes in the sections below.

In addition to the processing on the viral polyprotein, picornaviral $3C^{pro}$ have been shown to cleave host cell proteins during viral replication; many of these cleavages modulate transcription and translation in the infected cell (reviewed in Lloyd, 2006). However, at present, no common cellular target for all picornaviral $3C^{pro}$ has been identified. For this reason, the cellular targets of $3C^{pro}$ are mentioned in the specific sections below devoted to the $3C^{pro}$ of the different genera.

In addition to all these proteolytic activities, all the picornaviral $3C^{pro}$ also possess an RNA binding site, located on the opposite face of the molecule to that responsible for proteolysis (Allaire et al., 1994; Matthews et al., 1994; Skern et al., 2002). Specifically, the RNA binding site comprises parts of the N- and C-terminal helices as well as the part of the polypeptide chain that links the N- and C-domains. In entero- and rhinoviruses, this RNA binding site of $3C^{pro}$ or its precursor 3CD has been shown to bind to the clover-leaf structure at the 5′ end of the viral RNA to set up the replication complex on the genomic RNA (Andino et al., 1990; Zell et al., 2002). As this RNA binding sequence is extremely well conserved throughout the picornaviruses (Skern et al., 2002; Yin et al., 2005), a substance that interferes with the RNA binding site of one picornaviral $3C^{pro}$ may interfere with several other viruses across the different genera.

Comprehensive lists and descriptions of all $3C^{pro}$ inhibitors developed in the recent years have been compiled by Lall (Lall et al., 2004) and De Palma (De Palma et al., 2008).

6.2.1 Poliovirus $3C^{pro}$

The PV $3C^{pro}$ is the most specific of its class, cleaving solely at glutamine-glycine amino acid pairs. The structure of this $3C^{pro}$, determined by Mosimann et al. (Mosimann et al., 1997), revealed that glycine at the P1′ was required to turn the

polypeptide chain of the substrate away from the β-strand bI1 that effectively prevents the acceptance of any amino acid with a side-chain at the P1′ position. The specificity for the glutamine at P1 appears to be due to the presence of the uncharged residue histidine 161 that lies at the bottom of the P1 pocket. Importantly, histidine 161 is maintained in an uncharged state by hydrogen bonding of the hydroxyl group of tyrosine 138 with the nitrogen atom in the imidazole ring (Mosimann et al., 1997).

The specificity of PV $3C^{pro}$ whilst still bound to the $3D^{pol}$ protein is different from that of the mature $3C^{pro}$. Ympa-Wong et al. (Ypma-Wong et al., 1988) have shown that the 3CD protein is the form of $3C^{pro}$ responsible for cleaving the cleavages sites between capsid proteins VP2 and VP3 and between VP3 and VP1. However, the molecular and structural basis for this observation is not known. Even the recent determination of the structure of the 3CD precursor (Marcotte et al., 2007) failed to shed light on this issue.

Poliovirus $3C^{pro}$ has been reported to cleave a large variety of cellular proteins involved in transcription (Skern et al., 2002), translation (Lloyd, 2006; Perera et al., 2007; de Breyne, 2008), the ultrastructure of the cell (Joachims et al., 1995) and inflammation (Neznanov et al., 2005).

Sarkany and Polgar (Sarkany and Polgar, 2003) have examined in detail the catalytic mechanism of PV $3C^{pro}$ and note several unusual aspects whilst Gouvea and colleagues noted an unexpected behaviour of the enzyme towards chaotropic salts (Gouvea et al., 2006).

Given the need for a PV anti-viral outlined above, it is suprising that there are very few reports documenting specific inhibitors of PV $3C^{pro}$, in contrast to the situation with HRV (De Palma et al., 2008) and HAV (Lall et al., 2004).

6.2.2 Rhinovirus $3C^{pro}$

The groups of Matthews and Patick have made an extensive study of HRV $3C^{pro}$ and have developed many compounds active against these enzymes (Dragovich et al., 1998b; Hayden et al., 2003; Matthews et al., 1999; Matthews et al., 1994). One of the most effective compounds was AG7088 (later known as rupintrivir) (Matthews et al., 1999), a potent inhibitor of all HRV serotypes tested as well as some other related human enteroviruses (Binford et al., 2005). This compound showed promise in a phase II trial in the clinic (Hayden et al., 2003); however, in a further trial, the compound failed to diminish both viral load and disease severity and was thus not developed further (Patick, 2006; Patick et al., 2005).

The development of rupintrivir (shown bound to HRV2 $3C^{pro}$ in Fig. 6.2a) clearly illustrates that it is possible to develop a single inhibitor that is capable of inhibiting the replication of most rhinovirus serotypes (Binford et al., 2005). Such a property is not immediately obvious as the identity between serotypes can be as low as 50% (Duechler et al., 1987; Skern et al., 1985). However, analysis shows that many of the $3C^{pro}$ residues involved in binding rupintrivir are conserved in HRVs and human

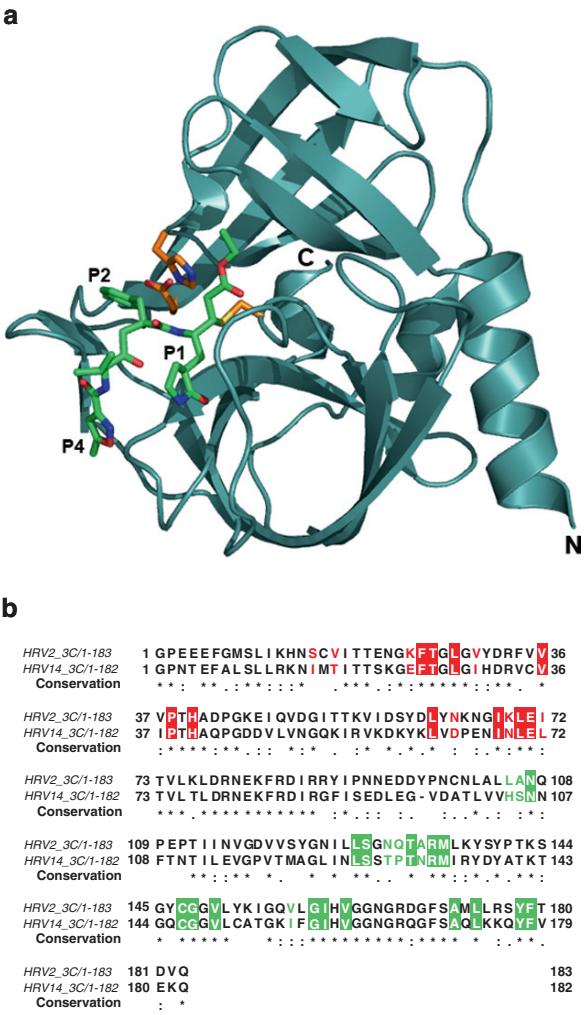

Fig. 6.2 The structure of HRV2 3C^{pro} (PDB 1CQQ) bound to the inhibitor rupintrivir and amino acid sequence conservation at the domain-interface. (**a**) Crystal structure of the HRV2 3C^{pro} covalently bound to the inhibitor rupintrivir. The protein is shown as a cartoon in blue with its catalytic triad shown as orange sticks. The inhibitor is depicted as a stick model, with carbon atoms in green, oxygen atoms in red, sulphur atoms in yellow, and nitrogen atoms in blue. The groups of the inhibitor mimicking the P4, P2 and P1 sites are labeled (generated with PyMOL DeLano, 2002). (**b**) Amino acid sequence alignment of HRV2 and HRV14 3C^{pro}. Residues lining the interface between the N- and C-terminal domain are colored red and green respectively. Conserved residues are shown in reverse video (generated with JalView Clamp et al., 2004)

enteroviruses, including PV (Binford et al., 2005). Interestingly, rupintrivir has been shown to be very effective against PV in cell culture (De Palma, communication at the XIV Meeting of the European Study Group on the Molecular Biology of Picornaviruses, Finland, Nov. 2006). In addition, Fig. 6.2a shows that rupintrivir makes contacts to both the N-terminal and C-terminal domains. Figure 6.2b shows that most of the residues at this domain interface are indeed conserved. As mentioned below, this is not the case for the domain interfaces of HRV 2A^{pro}.

What is the basis for the potency of rupintrivir? The electron acceptor of rupintrivir is a Michael group which forms a stable covalent adduct with the SH group of the active site nucleophile cysteine 147 of the 3C^{pro}. In Fig. 6.2a, the cysteine 147 side-chain is covalently attached to the inhibitor. The specificity of rupintrivir

derives from the moieties occupying the P1, P2 and P4 positions. At P1, a lactam ring (actually a cyclised analogue of glutamine) is present that makes favourable contacts with the residues of the P1 pocket (Fig. 6.2a) and may also stablise the free inhibitor in a conformation close to that found in the bound inhibitor (Matthews et al., 1999). The P2 residue of the inhibitor is a fluoro-phenyl moiety that mimics the large, bulky residue that is preferred by HRV $3C^{pro}$. Finally, the P4 moiety of the inhibitor is an iso-oxazole derivative that is well accepted by the small P4 pocket of these enzymes (Matthews et al., 1999). The position of each of the three moieties can be clearly seen in Fig. 6.2a.

Like PV $3C^{pro}$, HRV14 $3C^{pro}$ has been shown to cleave the p65-RelA subunit of NF-kappaB during replication (Neznanov et al., 2005).

6.2.3 *Coxsackievirus $3C^{pro}$*

In contrast to the other $3C^{pro}$, the first structural work on CV $3C^{pro}$ was to investigate its interaction with a synthetic RNA representing one of the stem-loops from the clover-leaf at the 5′ end of the viral RNA (Ohlenschlager et al., 2004; Zell et al., 2002). Interestingly, these observations show that the $3C^{pro}$ binds to a specific structure rather than a particular sequence of nucleotides. This suggests that inhibitors of CV $3C^{pro}$ binding may also inhibit the RNA binding of $3C^{pro}$ across the genera. Very recently, the structure of the CV $3C^{pro}$ itself was determined by Anand and co-workers using X-ray crystallography and the co-ordinates deposited in the database (see Table 6.1). This structure should aid in developing specific inhibitors for the CV $3C^{pro}$ and in understanding the interactions between the protein and the 5′ RNA clover-leaf.

Lee et al. have used rupintrivir as a lead compound to develop compounds effective against CVB2 $3C^{pro}$ (Lee et al., 2007).

6.2.4 *Hepatitis A virus $3C^{pro}$*

HAV $3C^{pro}$ differs strongly from the rhino- and enterovirus enzymes in that it prefers large residues at P4, smaller ones at P2, can accept glutamine or glutamate at P1 and has no specificity at the P' side. It can even accept such bulky residues as methionine or arginine at the P1′ position (Skern et al., 2002). Specificity at P4 in HAV $3C^{pro}$ is achieved through a larger pocket than in PV $3C^{pro}$; at P2, a small pocket is present for this residue that is absent in PV $3C^{pro}$. The P1 pocket residue of HAV is, as in HRV and PV $3C^{pro}$, an uncharged histidine residue, although the residues orienting this residue are not conserved (Skern et al., 2002). The difference at the P′ side is explained by the greater distance of the β-strand bI1 from the active site in the HAV $3C^{pro}$, removing the need for a glycine residue to turn the polypeptide chain of the substrate from this structure (Bergmann et al., 1997).

Several reports have described specific inhibitors for HAV $3C^{pro}$ (reviewed in Huitema et al., 2008; Lall et al., 2004; Yin et al., 2005). The enzyme has also been shown to cleave the host cell proteins poly(A) binding protein (PABP) and poly(C) binding protein II (Zhang et al., 2007a, b). Another unusual characteristic of this enzyme is that it has been reported to bind structures from the 5′ region of the viral RNA much more efficiently as a dimer than as a monomer (Peters et al., 2005).

6.2.5 Foot-and-Mouth Disease $3C^{pro}$

The structure of the $3C^{pro}$ of FMDV is similar to those of other picornaviral $3C^{pro}$, the major difference being the flexibility of a β-ribbon in the C-terminus of the molecule. This ribbon is capable of folding over the substrate binding site of the enzyme and providing residues involved in determining specificity. For example, the β-ribbon residue cysteine 142 appears to be involved in binding to the generally hydrophobic side chains of the P4 and P2 residues (Curry et al., 2007; Sweeney et al., 2007). The unusual ability of this enzyme to accept glutamate or glutamine equally at P1 remains unclear as the arrangement of the FMDV $3C^{pro}$ pocket accepting the P1 residue does not differ greatly from those of $3C^{pro}$ that discriminate against glutamate (e.g. HRV2 $3C^{pro}$ and PV $3C^{pro}$) (Birtley et al., 2005).

The FMDV $3C^{pro}$ has been shown to cleave a number of cellular proteins, including histone 3C (Falk et al., 1990; Tesar and Marquardt, 1990), the translation factors eIF4A (Li et al., 2001a) and eIF4G (Belsham et al., 2000) and gamma-tubulin (Armer et al., 2008).

6.3 Enteroviral and Rhinoviral 2A Proteinases

The first report that the 2A protein of PV contained a proteolytic activity cleaving between the C-terminus of VP1 and its own N-terminus was published by Toyoda et al. (Toyoda et al., 1986) in 1986. Subsequently, the same activity was demonstrated for the 2A proteins of HRV and CV (Liebig et al., 1993; Sommergruber et al., 1989). Inhibitor profiling and protein modelling showed that the $2A^{pro}$, like the $3C^{pro}$, are cysteine proteinases with a chymotrypsin-like fold. It seems likely that the $2A^{pro}$ arose by a duplication of the $3C^{pro}$ on the evolutionary precursor of the entero- and rhinoviruses. Despite this close relationship, there are however several clear differences between the $2A^{pro}$ and $3C^{pro}$ that preclude development of a single inhibitor for both proteinases. These differences concern the overall structures, the mechanisms and the specificity determinants. Thus, although the $2A^{pro}$ are closely related to the smaller serine proteinases such as streptomyces G protease B (SGPB) and α-lytic proteinase (Bazan and Fletterick, 1988; Petersen et al., 1999), structural analysis of the HRV and CV $2A^{pro}$ shows that their N-terminal domain lacks four β-strands present in the N-terminal domain of SGPB and $3C^{pro}$. In contrast, the

$2A^{pro}$ contain a zinc ion in the C-terminal domain, using a structural motif that is unique amongst the chymotrypsin-like proteinases (Petersen et al., 1999). In terms of mechanism, all $2A^{pro}$ use an aspartate residue as the third member of their active site triad; as mentioned above, glutamate or aspartate maintain this function in the $3C^{pro}$. Turning to specificity, the main specificity determinants for the $2A^{pro}$ are at P4, P2, P1′ and P2′, with several residues being accepted at the P1 position (Skern et al., 2002). This is in clear contrast to the well-defined P1 specificities observed in the $3C^{pro}$.

Rhino- and enteroviral $2A^{pro}$ have also been shown to cleave host cell proteins. In contrast to $3C^{pro}$, however, all $2A^{pro}$ have one cellular target in common, namely the cellular translation molecule eukaryotic initiation factor (eIF) 4G (Morley et al., 1997). This protein is present as two homologues, eIF4GI and eIF4GII (Gradi et al., 1998a). Cleavage of these homologues at a single site leads to the inability of the host cell to synthesise protein from its own capped mRNA. In contrast, viral RNA can still be translated from its internal ribosome entry site (IRES) and is even stimulated under these conditions (Ziegler et al., 1995). Individual $2A^{pro}$ have also been shown to cleave a variety of other cellular proteins. Some of these are discussed in the sections on the individual $2A^{pro}$ below.

The available structures for rhino- and enteroviral $2A^{pro}$ are listed in Table 6.1.

6.3.1 Poliovirus $2A^{pro}$

The PV $2A^{pro}$ was purified to homogeneity from infected cells just 2 years after its identification as a protease (Koenig, 1988). Subsequently, the recombinant protein was purified by affinity chromatography using maltose-binding protein and hexahistidine tags (Ventoso et al., 1998; Yalamanchili et al., 1997). In spite of these successes, it has not been possible to produce material of sufficient quantity and purity to allow structural work to proceed. This structure is perhaps the most important remaining target amongst the picornaviral proteases.

In contrast, much biochemical and molecular biological work has revealed a multitude of proteins that are cleaved by PV $2A^{pro}$. In addition to the eIF4G homologues, these cellular targets of PV $2A^{pro}$ include amongst others: the poly(A) binding protein PABP (Joachims et al., 1999), the TATA-binding protein (Yalamanchili et al., 1997), the catalytic subunit of the DNA-dependent protein kinase (Graham et al., 2004), proteins of the nuclear pore complex (Gustin and Sarnow, 2001; Park et al., 2008) and the protein gemin of the U snRNP assembly (Almstead and Sarnow, 2007).

The above list documents that the PV $2A^{pro}$ plays a major role in tailoring the infected cell to the needs of the virus. In addition, over and above its role in proteolytic processing, the PV $2A^{pro}$ has been shown to be involved in regulating stability, replication and translation of the RNA (Jurgens et al., 2006; Li et al., 2001b). PV $2A^{pro}$ is therefore truly a multifunctional enzyme. Along with the trans-dominant effect of certain $2A^{pro}$ mutations referred to above (Crowder and Kirkegaard, 2005), these properties make PV $2A^{pro}$ an excellent target for anti-viral substances.

6.3.2 Human Rhinovirus $2A^{pro}$

As mentioned above, the HRV genus contains over 100 antigenically distinct serotypes that can be grouped into the genetic clusters A and B. Comparison of the $2A^{pro}$ sequences from members of the different groups reveals an identity of only 40% (Sousa et al., 2006), about 10% less than between the $3C^{pro}$ of the different clusters (Argos, 1984). Nevertheless, for the $3C^{pro}$, these differences did not prevent the development of an inhibitor such as rupintrivir that was capable of inhibiting the $3C^{pro}$ of both groups and of several enteroviruses (De Palma et al., 2008). For HRV $2A^{pro}$, however, several lines of evidence suggest that the differences between the genetic group A and B $2A^{pro}$ will be sufficient to impede the development of a general inhibitor for HRV $2A^{pro}$. Indeed, over the years, we and others have made several observations that indicate differences in specificity and possibly also in mechanism between the $2A^{pro}$ of various rhinovirus and enterovirus $2A^{pro}$. These observations are summarised below.

First, the specificities of the HRV2 and HRV14 $2A^{pro}$, although not fully understood, appear to be different. Table 6.2 shows that the self-processing cleavage sites for the two enzymes clearly have only a few residues in common. Table 6.2 also shows the cleavage sites of HRV2 $2A^{pro}$ on the eIF4G homologues; it is believed that HRV14 $2A^{pro}$ cleaves the eIF4G homologues at these sites but it has not yet been demonstrated to do so. We have investigated this question by replacing the self-processing cleavage site of both HRV2 $2A^{pro}$ and HRV14 $2A^{pro}$ with that of the eIF4GI sequence shown in Table 6.2. The $2A^{pro}$ of HRV2 cleaved the eIF4GI site with the same efficiency as the wild-type sequence. In contrast, the eIF4GI cleavage site was refractory to HRV14 $2A^{pro}$ cleavage (Sousa et al., 2006). Using site-directed mutagenesis, we determined that lack of cleavage by the HRV14 enzyme was due to the presence of arginine at the P1 site in the self-processing reaction. HRV14 $2A^{pro}$ could not accept this residue whereas the HRV2 enzyme could (Sousa et al., 2006). This suggests that the substrate binding pockets for the P1 residue on these two $2A^{pro}$ differ considerably.

To examine this question more carefully, we examined the residues proposed to be involved in binding the P1 in HRV2 $2A^{pro}$, the only $2A^{pro}$ for which a high-resolution structure is available. In HRV2 $2A^{pro}$, the residue at the bottom of the P1 pocket appears to be Cys101 (Petersen et al., 1999); the corresponding residue in HRV14 would be A104. Substitution of A104 with cysteine did not, however, confer the

Table 6.2 Known cleavage sites of HRV2 and HRV14 $2A^{pro}$. The arrow indicates the cleavable bond. The cleavage sites on the eIF4G homologues have only been determined for HRV2 $2A^{pro}$ (Gradi et al., 2003; Skern et al., 2002)

Substrate	P5	P4	P3	P2	P1	↓	P1′	P2′	P3′	P4′
HRV2 (VP1–$2A^{pro}$)	Ile	Ile	Thr	Thr	Ala		Gly	Pro	Ser	Asp
HRV14 (VP1–$2A^{pro}$)	Asp	Ile	Lys	Ser	Tyr		Gly	Leu	Gly	Pro
eIF4G I	Thr	Leu	Ser	Thr	Arg		Gly	Pro	Pro	Arg
eIF4G II	Pro	Leu	Leu	Asn	Val		Gly	Ser	Arg	Arg

ability to recognise arginine at P1 by the HRV14 $2A^{pro}$ (Sousa et al., 2006). These results show that substrate recognition differs between enzymes of the two genetic groups and indicate that we do not at present know exactly which $2A^{pro}$ residues are involved in recognising the P1 residue. Insight into this question will require the determination of one or more structures of a $2A^{pro}$ from the genetic group B as well as a structure of a $2A^{pro}$ bound to an inhibitor or to a substrate analogue.

Another clear difference between HRV2 and HRV14 $2A^{pro}$ is their behaviour toward the inhibitor zVAM.fmk (benzyloxycarbonyl-Val-Ala-Met-fluoromethylketone). This inhibitor was developed after the observation by Deszcz et al. (Deszcz et al., 2004) that benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (zVAD.fmk) could inhibit replication of HRV2 by inhibiting the activity of the HRV2 $2A^{pro}$. zVAD.fmk was originally designed as a caspase inhibitor and is synthesised in an uncharged form to allow passage of the inhibitor through the cell membrane. Inside the cell, the methyl group on the aspartic acid residue is removed and the compound is able to inactivate caspases. Deszcz et al. (Deszcz et al., 2004) determined that the uncharged form of the inhibitor was responsible for the inhibition of the $2A^{pro}$. This agreed with previous data that had indicated that HRV2 $2A^{pro}$ can accept basic and hydrophobic residues at P1 but not acidic residues (Skern et al., 1991). Deszcz and colleagues (Deszcz et al., 2006) made use of this property to synthesise zVAM.fmk. This substance should inhibit HRV2 $2A^{pro}$ through the hydrophobic methionine residue but cannot be activated to an inhibitor of caspases. In cell culture experiments, zVAM.fmk could inhibit the replication not only of the genetic group A viruses HRV2 and HRV16, but also of the genetic group B virus HRV14 (Deszcz et al., 2006). This suggested a common mechanism of inhibition. However, further analysis showed that the HRV14 $2A^{pro}$ is inhibited in both intra- and intermolecular cleavage by zVAM.fmk whereas the HRV2 $2A^{pro}$ is only inhibited in intermolecular cleavage (Deszcz et al., 2006) (Sousa, C. and Skern, T., 2007, unpublished). Although these experiments indicate differences in HRV $2A^{pro}$, they do suggest that it may be possible to find inhibitors that inactivate a spectrum of $2A^{pro}$, even if the mechanism of inhibition differs. Second, these experiments clearly show that it is possible to design inhibitors that are specific for the $2A^{pro}$ but do not possess anti-caspase activity.

Another approach to finding compounds that inactivate the replication of a broad spectrum of HRVs would be to target genetic group A and B HRV separately. The above results suggest that a derivative of zVAM.fmk in which the methionine at the P1 position is replaced with arginine (zVAR.fmk, benzyloxycarbonyl-Val-Ala-Arg-fluoromethylketone) will inhibit the replication of genetic A group viruses but not genetic B group viruses. If this is true, it may be possible to specifically target genetic group B HRV in the same way.

A third example of differences between the genetic group A and B HRV is the difference in the onset of cleavage of eIF4GI and eIF4GII observed in cell culture (Gradi et al., 1998b; Seipelt et al., 2000; Svitkin et al., 1999). HRV2 $2A^{pro}$ has been shown to cleave the two homologues at about the same time during replication whereas the HRV14 $2A^{pro}$ clearly cleaves eIF4GI before eIF4GII. Neither the biological relevance nor the basis of this difference is understood,

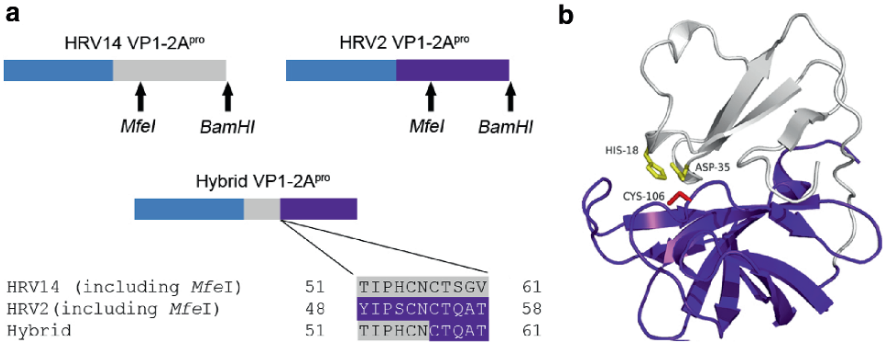

Fig. 6.3 Construction of an HRV14/2 hybrid 2A^{pro}. (**a**) *Mfe*I sites were introduced into the plasmids coding for either HRV2 or HRV14 VP1–2A^{pro} to enable exchange of restriction enzyme fragments encompassing an intact N- or C-terminal domain. An *Mfe*I and *Bam*HI fragment of HRV2 2A^{pro} was used to replace the C-terminal domain of HRV14 2A^{pro} and create a hybrid containing HRV14 VP1, the N-terminal 2A^{pro} domain of HRV14 and the C-terminal 2A^{pro} domain of HRV2. The sequence at the changeover is given. (**b**) The structure of HRV2 2A^{pro} (2HRV) is coloured according to its N-terminal and C-terminal domain. In the hybrid 2A^{pro}, the grey N-terminal domain will be exchanged by the respective HRV14 2A^{pro} domain. The catalytic triad (numbered according to HRV2 2A^{pro}) is coloured in yellow (His18 and Asp35) and red (Cys106) (generated with PyMOL DeLano, 2002)

although the difference could be a reflection of the above-mentioned differences in substrate specificity and inhibitor sensitivity.

The above examples document three phenotypic differences between the HRV2 and HRV14 2A^{pro}. We hypothesised that phenotypic differences might be determined by the property of one of the two domains of the 2A^{pro}. In an attempt to investigate this further, we constructed a hybrid 2A^{pro} containing the N-terminal domain of HRV14 and the C-terminal domain of HRV2. To achieve this, we noticed that an *Mfe*I restriction site could be introduced into the coding region of both enzymes at two of the four residues that form the zinc binding site. These two residues, asparagine 56 and cysteine 57 in HRV14 and asparagine 53 and cysteine 54 in HRV2 2A^{pro}, lie at the C-terminal end of the polypeptide linking the two domains (Fig. 6.3). Using the *Mfe*I and *Bam*HI sites, we introduced the C-terminal domain of HRV2 2A^{pro} (Fig. 6.3a, dark blue) into the HRV14 2A^{pro} (Fig. 6.3a, grey). Figure 6.3b shows the domain arrangement using the same colour scheme, based on the structure of HRV2 2A^{pro}.

We then tested the ability of this hybrid 2A^{pro} to carry out the self-processing reaction at the C-terminus of VP1 and its own N-terminus when either the HRV14 2A^{pro} or the HRV2 2A^{pro} cleavage site was present (Fig. 6.4). RNAs were transcribed *in vitro* and translated in RRLs. Figure 6.4 shows that the HRV14/2 2A^{pro} hybrid was unable to cleave either of the substrates. Even after 300 min of incubation, the only product produced was the uncleaved VP1-2A^{pro} running at 50 kDa; cleavage products running at 35 kDa (VP1) and 17 kDa (2A^{pro}) were absent (Fig. 6.4).

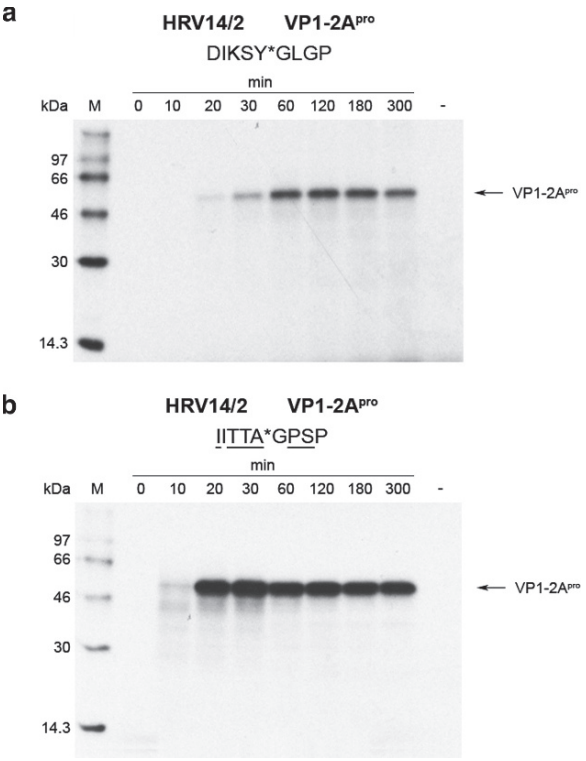

Fig. 6.4 Self-processing of HRV14/2 $2A^{pro}$ hybrid proteinase on the HRV14 (**a**) and HRV2 (**b**) cleavage site. The amino acids in the cleavage sites are shown above the gels. Differences between the sites are underlined in the HRV2 sequence (**b**). Rabbit reticulocyte lysate was programmed in the presence of ^{35}S methionine with *in vitro* transcribed RNA coding for HRV14/2 VP1–$2A^{pro}$ (10 ng/µl) and incubated at 30°C. Negative controls were prepared by adding water instead of RNA. 10 µl aliquots were taken at the given time points and put on an icecold mix of 25 µl 2x Laemmli sample buffer, 15 µl H_2O and 1 µl unlabeled methionine/cysteine (20 mM). Viral proteins were then separated by SDS-PAGE on 17.5% gels and visualized by fluorography. Protein standards (M) in kDa are given on the left

The hybrid $2A^{pro}$ is clearly inactive. What might the reasons for this be? To answer this question, we examined the nature of the residues that comprise the substrate binding region at the interface of the N- and C-terminal domains in HRV2 and HRV14 $2A^{pro}$. Figure 6.5 shows that only 6 of the 20 residues lining the interface of the N- and C-terminal domains are identical in the $2A^{pro}$ of the two genetic groups. Furthermore, three of these are involved in generating the active site triad. In contrast, this is not the case for the picornaviral $3C^{pro}$; in this enzyme, the opposite is true and most of the residues lining the domain interface are conserved (Fig. 6.2). These differences in $2A^{pro}$ between the genetic groups appear from the experiment in Fig. 6.4 to affect substrate specificity and will have to be born in mind when designing inhibitors that should possess a broad spectrum of activity

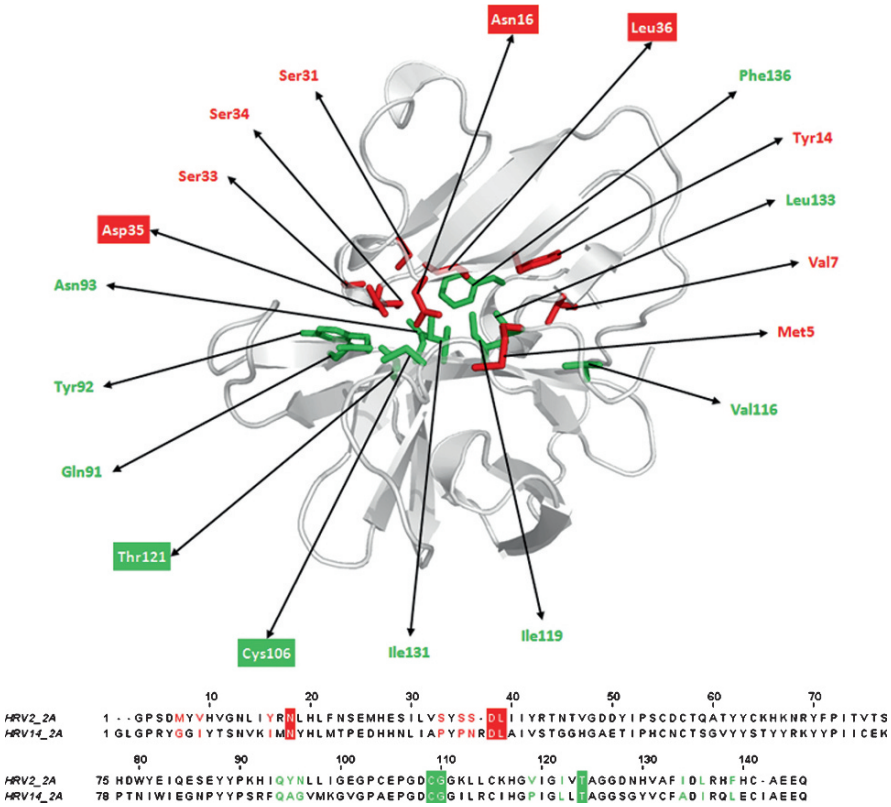

Fig. 6.5 Residues situated at the interface between the N-terminal and C-terminal domain of HRV2 2A^{pro}. (**a**) The 2A^{pro} molecule is shown as a grey ribbon at half-transparency to allow all domain interface residues to be visualised. Residues at the domain interface are shown as sticks. (**b**) Alignment of HRV2 and HRV14 2A^{pro} sequences. N-terminal domain residues at the domain interface are coloured red, C-terminal domain residues at the domain interface are in green. Reverse video in the labels and the alignment indicates conserved residues between HRV2 and HRV14 2A^{pro}. (structure generated with PyMOL DeLano, 2002) and JalView (Clamp et al., 2004)

against HRV 2A^{pro}. Furthermore, this observation again underlines the need for the determination of the structure of a 2A^{pro} from a genetic group B virus.

The section on the PV 3C^{pro} outlined the differences in structure and function between the 3C^{pro} and its precursor 3CD. For entero- and rhinoviral 2A^{pro}, there is the similar possibility that the adjacent protein on the polyprotein, 2B, can influence the properties of the 2A^{pro}. We have observed such an effect on the processing by 2A^{pro} in RRLs. Figure 6.6 shows that the presence of a single mutation, the substitution of isoleucine for asparagine at residue 94, four amino acids from the C-terminus of 2B, is capable of completely abrogating the activity of HRV14 2A^{pro}. Cleavage by wild-type 2A^{pro}-2B takes place over a period of 60–180 min, with the cleavage products VP1 (40 kDa) and 2A^{pro}-2B (28 kDa) being visible even after 20 min (Fig. 6.6a). In contrast, only the uncleaved precursor is visible in the I94N

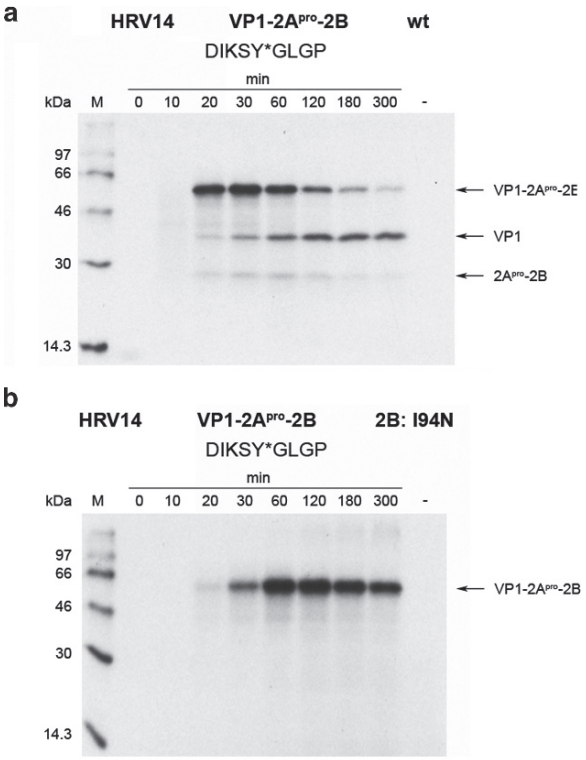

Fig. 6.6 Effect of a mutation at the C-terminus of 2B on the self-processing of HRV14 2A^{pro} on the VP1–2A^{pro}-2B polyprotein. Rabbit reticulocyte lysate was programmed with *in vitro* transcribed RNA coding for HRV14 VP1–2A^{pro}-2B wild-type (**a**) and I94N (**b**). The experiment was performed as described in the legend to Fig. 6.4

mutant (Fig. 6.6b). It will be of interest to investigate whether the differences in the inhibition profiles seen with zVAM.fmk on HRV replication may derive from differences in the properties of the 2AB precursor.

6.3.3 Coxsackievirus 2A^{pro}

One of the missing pieces in our understanding of the 2A^{pro} is the mechanism of self-processing. We attempted to gain information on this subject by using NMR to investigate the structure of inactive CVB4 2A^{pro} containing 8 amino acids of VP1 at the N-terminus. The enzyme was inactivated to prevent loss of the eight amino acids of VP1 by substituting the active site residue Cys110 with alanine. CVB4 2A^{pro} was used because we were unable to obtain soluble protein with the analogous HRV2 protein. We were able to assign ^{13}C and ^{15}N signals for most of the CVB4 2A^{pro} (Baxter et al., 2006). Unfortunately, however, NMR signals could not be assigned for the last C-terminal eight amino acids of VP1 and first eight N-terminal amino acids of the 2A^{pro}. The reason for this inability was determined by performing studies on the conformational dynamics of the CVB4 2A^{pro}. ^{15}N NMR relaxation measurements clearly indicated that the N-terminus of the enzyme and

its VP1 extension were slowly and reversibly moving in and out of the active site, resulting in a broadening of these signals beyond detection (Baxter et al., 2006).

Structural information on the mechanism of self-processing by $2A^{pro}$ will be vital for the development of substances active against CV. We are continuing to investigate this question using X-ray crystallography on CV $2A^{pro}$ bearing a range of different VP1 extensions.

As mentioned above, the CV $2A^{pro}$ cleaves the myospecific protein dystrophin during infection (Badorff et al., 1999). This cleavage has been detected in a biopsy from a patient with dilated cardiomyopathy (Badorff and Knowlton, 2004). Encouragingly, this cleavage could be inhibited *in vitro* by a substance corresponding to the CV $2A^{pro}$ cleavage site on dystrophin (benzyloxycarbonyl-LeuSerThr-Thr–fluoromethylketone) (Badorff et al., 2000). However, the potential of this and related compounds has not yet been followed up.

6.4 Aphthoviral L Protease

6.4.1 Foot-and-Mouth Disease Virus L Protease

The Leader protease (L^{pro}) of FMDV was demonstrated to be able to free itself from the growing polypeptide chain by cleavage at its own C-terminus and the N-terminus of VP4 by Beck and Strebel (Strebel and Beck, 1986). Subsequently, Gorbalenya et al. (Gorbalenya et al., 1991) proposed that L^{pro} was a papain-like cysteine protease. This proposal was further supported by the ability of E64, a classical inhibitor of papain-like proteases, to inhibit L^{pro} (Kleina and Grubman, 1992) and modelling of the L^{pro} based on structural comparisons with those of papain and papain-like cathepsins (Skern et al., 1998). The determination of the three-dimensional structure of L^{pro} in 1998 (Guarné et al., 1998) confirmed these findings, revealing that L^{pro} has indeed a classical, if somewhat reduced, papain-like fold. In contrast to papain, however, L^{pro} has no pre-pro domain at the N-terminus; instead, it possesses a C-terminal extension (CTE) of 18 amino acids protruding from the globular domain of the enzyme. The CTE is thought to be flexible enough to reach back into the active site of the enzyme to allow intramolecular self-processing at the junction of the C-terminus with VP4. In the crystal structure, however, the CTE of one molecule was found in the active site of the adjacent one and vice-versa, thus presenting a picture of an intermolecular cleavage in the form of a homodimer.

Like the $2A^{pro}$ of rhino- and enteroviruses, L^{pro} also cleaves the eIF4G homologues, again inhibiting the translation of the host cell mRNA (Devaney et al., 1988; Gradi et al., 2004). However, attempts to find other targets for this enzyme in the infected cell have not been fruitful (Foeger, N., Hampoelz, B. and Skern, T., 2003, unpublished). Recent work has demonstrated that a portion of the L^{pro} in the infected cell translocates to the nucleus (de Los Santos et al., 2006). This raises the intriguing possibility that L^{pro} may have targets in this cellular compartment.

The inhibition of L^{pro} processing and FMDV replication shows that L^{pro} is a suitable target for anti-viral therapy (Kleina and Grubman, 1992). In addition, a recombinant FMDV lacking the L^{pro} sequence did not spread in an infected animal and appeared to be extremely sensitive to the interferon response, probably as a direct result of the absence of eIF4G cleavage (de Los Santos et al., 2006). E64, although well-tolerated by animals, is nevertheless a general inhibitor of many papain-like cysteine proteinases. It is therefore necessary to develop inhibitors that are more specific for L^{pro}.

Attempts to generate specific peptido-mimetic inhibitors of L^{pro} have however been hampered by the inability to define a consensus sequence for L^{pro} cleavage. Table 6.3 shows the cleavage sequences of the three known in vivo substrates of L^{pro}. At first glance, the sequences appear quite disparate and, on reflection, intensify the interest in the question why L^{pro} does not appear to cleave more proteins in the infected cell. However, close examination reveals that the cleavage sites imply specificity at P2 for leucine or valine and a requirement for a basic amino acid at P1, P1′ or P2′. If a basic residue is not present at one of these positions, then a small residue such as glycine or serine at P1 or P1′ or alanine or threonine at P2′ is required. Mutational analysis confirmed this interpretation, showing that L^{pro} accepted poorly substrates containing phenylalanine at P2 and did not cleave well on substrates lacking basic residues at P1, P1′ and P2′ (Glaser et al., 2001). These specificity determinants of L^{pro} are extremely unusual. It is indeed quite exceptional that a protease is able to cleave before as well as after a basic residue. This unusual property may however be advantageous in efforts to develop specific inhibitors of L^{pro}.

As with $2A^{pro}$, little is known about the mechanism of self-processing in L^{pro}. To investigate this question, we again turned to NMR, believing that this method could show how the CTE moves in and out of the active site. We also felt that measuring the structure of L^{pro} in solution would prevent the formation of the dimer observed in the crystal. We were therefore very surprised when the full-length L^{pro} behaved in solution as it did in the crystal structure by forming a homo-dimer (Cencic et al., 2007). Again, the dimer was formed by the insertion of the CTE of one molecule into the active site of the second molecule and vice versa and appeared to be further stabilised by residues at the interface of the two globular domains.

We confirmed that the C-terminus of the L^{pro} was responsible for the formation of the dimer by analysing the NMR signals of a variant of L^{pro} (termed sL^{pro}) lacking six amino acids at the C-terminus. sL^{pro} behaved as a monomer in solution, with the truncated 12 amino acid long CTE indeed showing a great degree of flexibility.

To examine the strength of dimerisation, we first added sodium chloride to the NMR sample; however, the dimer remained stable up to 2M sodium chloride, indicating that the L^{pro} dimer is a very stable one. To investigate the strength of the

Table 6.3 Known cleavage sites of L^{pro}. The arrow indicates the cleavable bond (Gradi et al., 2004; Skern et al., 2002)

Substrate	P5	P4	P3	P2	P1	↓	P1′	P2′	P3′	P4′
Polyprotein (L^{pro}–VP4)	Gln	Arg	Lys	Leu	Lys		Gly	Ala	Gly	Gln
eIF4G I	Phe	Ala	Asn	Leu	Gly		Arg	Thr	Thr	Leu
eIF4GII	Leu	Leu	Asn	Val	Gly		Ser	Arg	Arg	Ser

dimer further, we then turned to size exclusion chromatography, as this approach requires much lower amounts of protein than NMR. With this method, we were able to estimate an upper limit of 500 nM for the K_D of L^{pro} dimer dissociation. This value suggests that L^{pro} may be present as a dimer even at the low concentrations of L^{pro} found in the infected cell and thus be relevant for biological activity. This observation represents an interesting avenue of future research.

In a further attempt to examine the self-processing reaction, we decided to examine the properties of a mutant of L^{pro} in which residue leucine 200 (i.e. the last but one residue at the C-terminus corresponding to the P2 position of the substrate) had been replaced by phenylalanine. We had previously shown that this mutant was severely impaired in self-processing (Kuehnel et al., 2004; Mayer et al., 2008) and believed that the presence of the phenylalanine would impair or even completely eliminate dimer formation. This would allow us to examine the properties of a monomeric form of L^{pro} that contains a full-length CTE that could still be expected to move in and out of the active site, affecting the NMR signals of atoms in this area and providing information on self-processing.

To this end, we expressed and purified the L200F mutant of L^{pro} as described (Kirchweger et al., 1994) and examined its behaviour on size exclusion chromatography. Figure. 6.7 shows that this protein indeed behaves as a monomer, eluting at about 68 ml, the same position as sL^{pro}. In contrast, the wild-type L^{pro} elutes at about 58 ml, corresponding to the dimeric form. We are now investigating the behaviour of the CTE of the L200F mutant by NMR. Although we have not yet shown that the

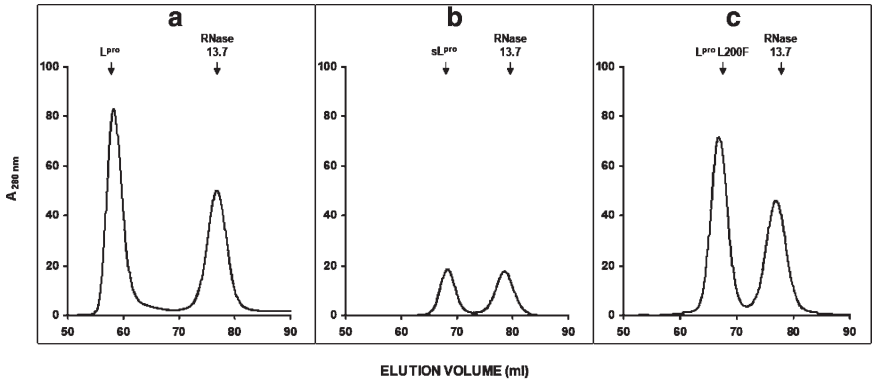

Fig. 6.7 Size exclusion chromatography of the FMDV Leader protease (L^{pro}), the shortened form (sL^{pro}) and a mutant Leader protease (L^{pro} L200F) on a HiLoad 16/60 Superdex 75 prep grade. Experiments were performed as described by Cencic et al. (Cencic et al., 2007). Ribonuclease A (RNase) with a molecular mass of 13.7 kDa was used as a reference. The elution profiles of the following combinations are shown: (**a**) L^{pro} (1 mg) and RNase (3 mg); (**b**) sL^{pro} (1 mg) and RNase (3 mg); (**c**) L^{pro} L200F (1 mg) and RNase (3 mg). Samples were applied at a flow rate of 1 ml min^{-1} in buffer A (50 mM NaCl, 50 mM Tris-HCl pH8, 1 mM EDTA, 5 mM DTT, 5% Glycerol). The apparent molecular masses in kDa are shown above the peaks. L^{pro} elutes at a volume corresponding to a molecular mass of about 40 kDa, indicating the formation of a dimer; in contrast, sL^{pro} elutes corresponding to a molecular mass of about 20 kDa, indicating a monomeric state. L^{pro} L200F elutes at a volume corresponding to a molecular mass of 20 kDa and thus resembles a monomer

dimer is present in vivo, its strength suggests that it may be involved in its biological activity. If so, the prevention of dimerisation by small molecular compounds may be another approach to interfering with the replication of FMDV.

6.4.2 *The Interaction of HRV2 2A^{pro} and L^{pro} with the Cellular Protein eIF4GI*

The above discussion concentrated on the feasibility of developing classical inhibitors of the proteolytic activity of the picornaviral proteases. In the last few years, it has however become clear that both the HRV2 2A^{pro} and the L^{pro} interact with the cellular protein eIF4G using exosites (i.e. regions of the protein away from the canonical substrate binding site). This was first observed with mutations in the CTE of L^{pro} lying ca 40 Å from the active site that interfered with the cleavage of eIF4GI without affecting the self-processing reaction (Foeger et al., 2002). Subsequently, it was shown that residues 183–195 of the CTE bound directly to the region 645–657 of eIF4GI, about 20 residues upstream of the L^{pro} cleavage site between glycine 674 and arginine 675 (Table 6.3) (Foeger et al., 2002; Foeger et al., 2005). A similar situation was also observed with HRV2 2A^{pro}. Here, residues 17–35 of the N-terminal domain, lying outside of the canonical substrate binding domain, were found to affect eIF4GI cleavage without impairing the self-processing reaction. Site-directed mutagenesis and binding experiments showed that this region of the HRV2 2A^{pro} interacted directly with a region of eIF4GI between residues 600 and 674, some distance from its actual cleavage site between amino acids arginine 681 and glycine 682 (Table 6.2) (Foeger et al., 2003). Thus, in spite of the fact that HRV2 2A^{pro} and L^{pro} are completely different enzymes in terms of structure, mechanism and specificity, both have evolved a mechanism to enable rapid cleavage of a molecule that is central to protein synthesis. At present, no host proteins have been identified as binding to eIF4G in this same manner. Therefore, inhibition of these specific interactions may also represent drug targets to fight infections with these viruses. The recent report of an inhibitor that can inhibit the interaction of eIF4GI with its binding partner eIF4E strengthens this notion (Moerke et al., 2007). The idea of inhibiting the HRV2 2A^{pro} or L^{pro} interaction with the eIF4G homologues is in line with a recent review covering the general idea of inhibiting protein-protein interactions for therapeutic purposes (Wells and McClendon, 2007). In the future, it will therefore be important to identify further specific interactions of picornaviral proteases with viral and host proteins in order to widen our options in fighting picornaviral infections.

6.5 Conclusions

Picornaviral proteinases represent attractive anti-viral targets for two reasons. First, they are absolutely required for the replication of the viruses, second, their substrate specificities differ from proteases of the host cell. Despite intensive research over

the last 25 years, however, anti-virals to treat picornaviral diseases have not been approved. The most promising of all candidates, rupintrivir, unfortunately failed to reduce viral severity in the clinic and development was halted. This result implies that treatment of picornaviral infections may require two anti-viral substances attacking different viral targets for full inhibition. Although the development of a second substance as promising as rupintrivir may seem a daunting task, the work described here outlines several new avenues for the generation of such substances. Above all, the L^{pro} of FMDV and $2A^{pro}$ of rhino- and enteroviruses have not been fully examined. In addition, the possibility of inhibiting RNA binding by $3C^{pro}$ requires further investigation. All of these approaches need to be examined soon if anti-viral therapy is to play a role in the end-game of poliovirus eradication.

Acknowledgements This work was supported by a grant from the Austrian Science Foundation (P20889) to T.S.

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