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Verfasserin:	Stephanie Schertler
Matrikel-Nummer:	0002230
Studienrichtung (lt. Studienblatt):	A419 Chemie Diplom
Betreuerin / Betreuer:	Ao. Univ.- Prof. Dr. Timothy Skern
Wien, am	

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1. INTRODUCTION

1.1. Picornaviruses

1.1.1. General information

Picornaviruses (family: picornaviridae) are non-enveloped viruses with a single-stranded RNA genome of positive sense and an icosahedral capsid. The name picornavirus derives from the Greek word *pico* meaning very small, and RNA referring to their ribonucleic acid genome. The name literally means small RNA virus. The family of picornaviridae has been object of many studies; the disease caused by one type of picornavirus, foot and mouth disease virus (FMDV), was already described in 1514 (Fracastorius, 1546). They are an interesting and important target for various studies as picornaviruses are the causative agents of several human and animal diseases.

Picornaviridae		
genus	species	serotypes
Rhinovirus	Human rhinovirus (HRV)	102
	Bovine rhinovirus (BRV)	3
Aphthovirus	Foot- and- mouth- disease virus (FMDV)	7
	equine rhinitis A virus (ERAV)	1
Enterovirus	Poliovirus (PV)	3
	Coxsackie virus A (CVA)	23
	Coxsackie virus B (CVB)	6
	Echovirus (EV)	30
	Human enterovirus (HEV)	4
	Bovine enterovirus (BEV)	2
	Porcine enterovirus A (PEV-8)	1
	Porcine enterovirus B (PEV-9, PEV-10)	2
	Swine vesicular disease virus (SVDV)	1
Parechovirus	Human parechovirus (HPEV)	2
Cardiovirus	Encephalomyocarditisvirus (EMCV)	1
	TEMV, VHEV	2
Hepatovirus	Human hepatitis A virus (HAV)	1
	Simian hepatitis A virus (SAV)	1
Teschovirus	Porcine teschovirus (PTV)	10
Kobuvirus	Aichi virus	1
Erbovirus	equine rhinitis B virus (ERBV)	1

Tab. 1 genera of the picornaviridae family

According to taxonomy there are 9 genera of classified picornaviruses, which can be seen in table 1 (Stanway *et al.*, 2005). More than 200 serotypes among the several genera of picornaviruses are known. These can be classified according to their physiological properties and their phylogenetic relationships, which is based on alignments of their nucleotide sequence.

According to Baltimore's classification system, picornaviruses are class IV as their genome is a single stranded RNA molecule of positive sense with a length of 7,2kb (HRV14) to 8,5kb (FMDV). As in all positive sense RNA viruses the genomic RNA is infectious. However, intact viral particles are 1×10^6 - fold more infectious. On both ends of the genome, there is an untranslated region (UTR). The 5' UTR is 600 to 1200 base pairs long and contains a higher ordered structure, the internal ribosome entry site (IRES), which is responsible for cap independent internal initiation of translation. The 3' UTR is only 50 to 100bp long and plays a role in the synthesis of the negative strand. The rest of the genome codes for a single polyprotein of 2100 to 2400 amino acids. On the 5' end there is no cap-structure, but a small virally encoded protein, VPg (*virus protein, genome linked*), which contains approximately 23 amino acids and is covalently attached to the RNA. The 3' end is polyadenylated. The genome has one open reading frame. The 5' region of the genome encodes for structural proteins, whereas the 3' region encodes for non-structural proteins (Kitamura *et al.*, 1981; Racaniello *et al.*, 1981; Stanway *et al.*, 1983) (Fig.1).

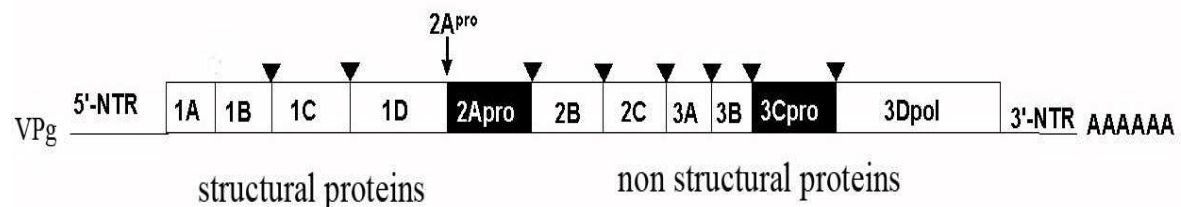


Fig.1: Genome of picornaviruses

The figure shows the single-stranded positive sense RNA genome of picornaviruses. On both ends there is an UTR, the rest of the genome codes for a single polyprotein. On the 5' end VPg, a small virally encoded protein, is attached, the 3' end is polyadenylated. The black shading marks 2A^{pro} and 3C^{pro} the two proteases that cleave the polyprotein into segments.

The genome has a length of 2500nm, whereas the viral particles have a diameter of only 27 to 30nm. This means that the genome has to be packed very tightly into the icosahedral capsid. The capsid of the viral particle consists of a tightly packed arrangement of 60 protomers. Each protomer consists of four different polypeptides (VP1, VP2, VP3, and VP4). VP2 and VP4 derive from the cleavage of the primary protomer VP0 (Arnold & Rossmann, 1990). In each

of the 12 axes of symmetry a pentamer consisting of 5 protomers is arranged. This results in the icosahedral capsid in which the viral RNA genome is packed (Fig.2).

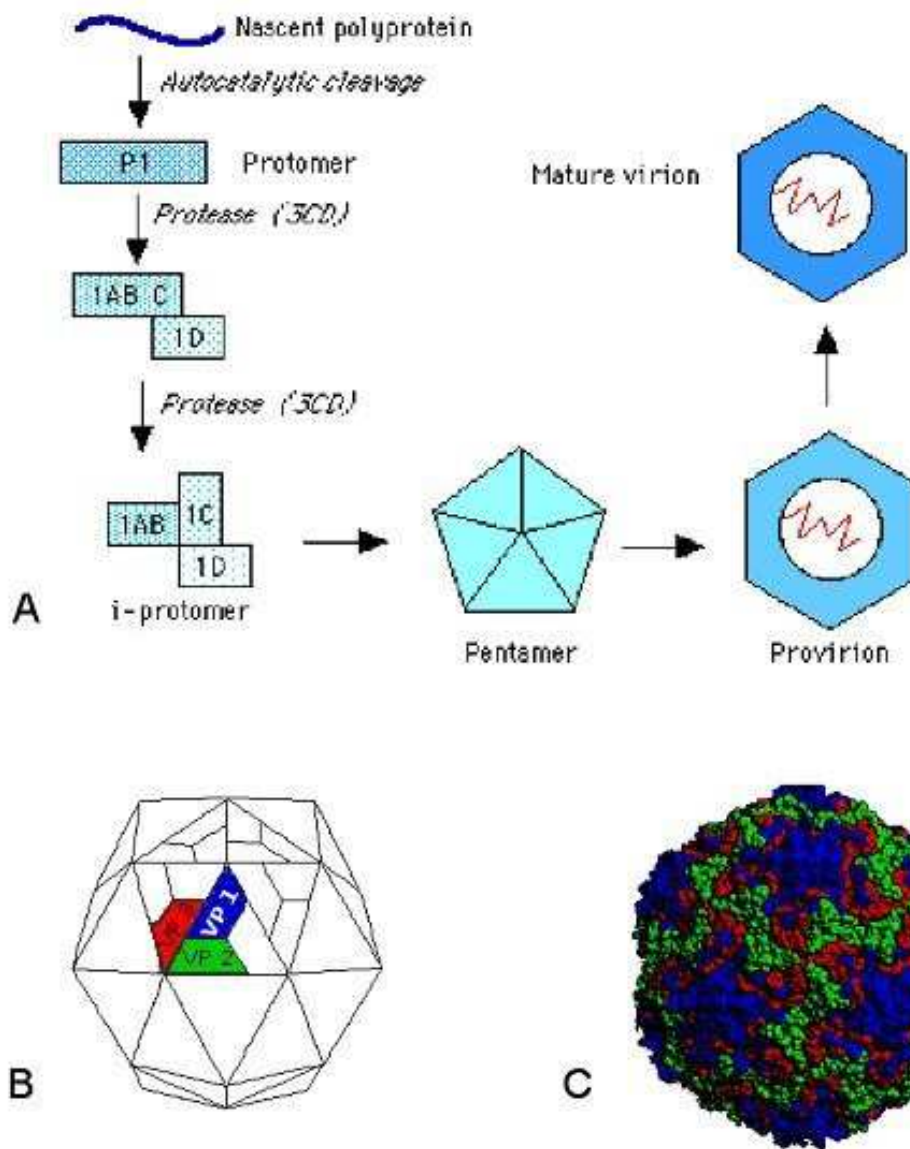


Fig.2: Assembly of the viral particle

A shows the assembly of the viral particle from the nascent polyprotein to the mature virion. Graph B demonstrates the packaging of the viral proteins VP1, VP2 and VP3 in a picornaviral capsid. The x-ray structure of the picornavirus HRV14 is given in C. VP1 is marked in blue, VP2 in green and VP3 is marked in red. VP4 is only visible on the inside of the particle. A has been taken from [1], picture B and C from [2].

Viruses have to keep their genomes small and have evolved different strategies to do so. Picornaviruses have two major strategies to increase the capacity of their small genome. First, they are translated using internal initiation of translation as their 5' UTR contains an IRES (Pelletier *et al.*, 1988; Jang *et al.*, 1988). Secondly, their positive sense RNA genome contains a single ORF that is translated into a single polyprotein. This saves space as only one mRNA

is required for several proteins. Following translation the polyprotein is processed into the viral proteins by the viral proteases 2A, 3C and L. More details on picornaviral proteases are given in chapter 1.3.

The replication of picornaviruses takes entirely place in the cytoplasm. The first step in viral replication is the attachment of the virion to the membrane of the host cell. For attachment the various serotypes use different receptors. It is believed that in most picornaviruses the virus enters the cell by receptor-mediated endocytosis. However, some picornaviruses, e.g. poliovirus enter via structural rearrangements (Zeichardt, 1985; Jackson *et al.*, 1996; Curry *et al.*, 1995; Willingman *et al.*, 1989). In case of receptor-mediated endocytosis, the viral particle is drawn into the cytoplasm when the virus- receptor complex is invaginated into the cell and then closed off into a clathrin coated vesicle. These clathrin coated vesicles fuse with intracellular vesicles forming endosomes. Inside the vesicles, a significantly lowered pH (pH < 5) can be found. The uncoating of the virus is pH dependent as the low pH results in a conformational change of the capsid (Madhus *et al.*, 1984). Due to this conformational change, the VP4 is lost and the surface becomes more lipophilic. The viral RNA, to which the VPg is covalently attached, is released into the cytosol through pores (Giranda *et al.*, 1992). Subsequently, the translation of the polyprotein starts. The positive sense viral RNA is used as a mRNA and translated into a polyprotein of about 220kDa. After the *cis* processing in which the viral proteases cleave the polyprotein into segments, RNA polymerase is released. This polymerase uses the positive sense strand as a template to create the complementary negative strand. To produce the negative strand, the VPg is esterified with uridine. The VPg-pUpU complex interacts with the poly-A tail and can be used as a primer for the polymerase. The negative strand then forms a replicative intermediate in the endoplasmic reticulum. This multistranded replicative intermediate can be used to replicate several positive sense RNAs concomitantly. (Richards *et al.*, 1984; Pata *et al.*, 1995) These positive sense RNAs are again used for the translation of the viral proteins and the replication of the negative sense strands.

The newly synthesised capsid proteins arrange first to protomers and then to pentamers. Then, the positive sense RNA condenses to a compact core around which 12 pentamers are arranged to form the viral capsid. The mature virion is created after the processing of VP0 into VP2 and VP4. Following this, the readily packed viral particles accumulate in the cell and are released. The release of the virus from the cell is not fully understood. As most non-enveloped viruses are dependent on disintegration of the host cell, the mature virus is probably accumulated until the cell is lysed. However, some form of cellular exocytosis

(Sandoval & Carrasco, 1997) or induced apoptosis (Rasilainen *et al.*, 2004) may play a role as well. The steps of the replication cycle are summarized in Fig.3.

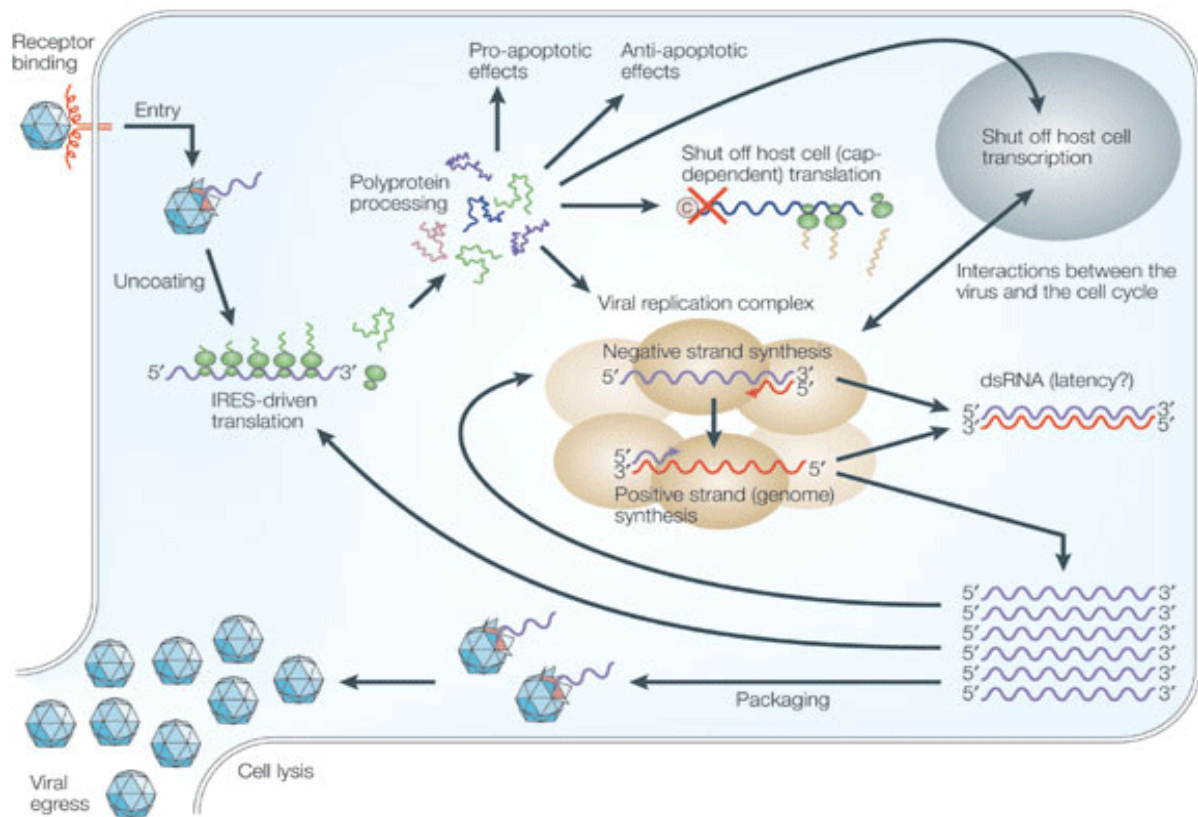


Fig.3: The viral replication cycle of a picornavirus

The figure demonstrates the viral life cycle of a picornavirus starting with binding to the receptor, followed by the entry of the cell and uncoating of the virus. The virus then uses the cellular machinery to translate its polypeptide. The cleavage of the two isoforms of eIF4G by 2A^{pro} then leads to a shut-off of host cell translation while the viral proteins are still translated through a cap independent mechanism. The viral life cycle is then completed by packaging and release of the viral particle. Fig. 3 has been taken from Whitton *et al.*, 2005)

1.1.2. Rhinoviruses

Being the main causative agent of common cold, the 102 serotypes of human rhinovirus (HRV) are the most common infective agents in humans (Monto *et al.*, 1987; Rueckert *et al.*, 1996). The name is derived from the Greek word *rhino* meaning nose.

Rhinoviruses are transmitted either directly from person- to- person via airborne infection by aerosols of respiratory droplets, or indirectly via respiratory droplets deposited on hands which are transported by the fingers to the nose or the eyes (Hendley & Gwaltney, 1988). Rhinoviruses occur worldwide and cause infections of the upper respiratory tract, where they affect the epithelial layer of the nose. Their ideal growth temperature is 33°C, which

corresponds to the temperature inside the nose. Therefore, rhinoviruses rarely cause infections of the lower respiratory tract, as they grow poorly at 37°C. The incubation period for rhinovirus infections is only 8- 10 hours. The infection is normally harmless. Complications are mainly due to secondary bacterial infections which may cause problems in children and old people (Miller *et al.*, 2007). However, rhinovirus infections are an economic factor as they cause days absent from work (Bramley *et al.*, 2002; Fendrick *et al.*, 2003). Protection from infection is maintained by antibodies in the respiratory tract (Couch, 1996). However, it has to be remarked that these antibodies do not circulate lifelong, but are short-living. A vaccine against rhinoviruses does not exist as there is little- to no cross- protection between the serotypes.

The various serotypes of HRVs can be separated into different groups of the rhinovirus genus. The first classification is based on an alignment of the nucleotide sequences of the VP4/VP2 region (420nt). HRV- A consists of 74 serotypes, while 25 serotypes can be classified as HRV- B.(Savolainen *et al.*, 2002b) HRV- 87 could be identified as human enterovirus 87 by VP4- based molecular diagnosis (Savolainen *et al.*, 2002a; Ishiko *et al.*, 2002). HRV 1 is separated into two subtypes, HRV-1A and HRV-1B. A second classification scheme is based on the receptors used by the different serotypes. HRVs that use the LDL- receptor are members of the so-called minor group, whereas HRVs using ICAM- receptors belong to the major group. (Schober *et al.*, 1998)

In this thesis, the proteins of two serotypes of HRV, HRV2 and HRV14, were examined. According to the classification systems described above, HRV2 is a member of the HRV- A group and a minor group virus. HRV14 on the other hand belongs to HRV- B and the major group. Thus the two serotypes differ in their sequence and the type of receptor they use (Duechler *et al.*, 1993).

1.1.3. Polioviruses

Poliovirus (PV) belongs to the genus enterovirus and is the causative agent of poliomyelitis. It was first isolated in 1908 by Landsteiner and Popper (Landsteiner & Popper, 1909), but first cases of poliomyelitis were already documented in prehistoric Egyptian drawings. It is one of the most well-characterized viruses and often used as a model system for RNA viruses.

There are three serotypes of poliovirus, PV1, PV2 and PV3. The length of the genome of each is about 7500 bp. The capsid proteins differ between the three serotypes. PV1 is the most common serotype in nature. There is evidence that PV emerged from C- cluster coxsackie A viruses through a mutation in the capsid. This mutation probably resulted in a change of the used receptor and a change of the pathogenicity of the virus. (Ping *et al.*, 2007)

All serotypes of PV use CD155 as receptor, which can be found in the cells of humans, higher primates and old world monkeys. Poliovirus naturally infects only humans. PV is an enterovirus and is transferred via the faecal- oral route, which means that the virus is found in the faeces and can replicate in the alimentary tract when ingested. This is only possible as PV is acid-stable and not destroyed in the acidic surroundings of the alimentary tract.

The incubation period of PV lasts for 7-14 days. The virus is replicated in the intestinal tract and then attacks the local lymph nodes and enters the bloodstream (viremia). In more than 90% of the cases the viremia is only transient and the poliovirus infection is asymptomatic. In about 5% of the cases the virus spreads and replicates in other tissues such as brown fat, muscle and the central nervous system (CNS). This sustained infection results in an aseptic meningitis which is accompanied by minor symptoms such as fever, headache and a sore throat, however there are no signs of paralysis. In only 1% of the infections the virus enters the CNS and replicates in motor neurons within the spinal cord, the brain stem and the motor cortex. This results in destruction of the motor neurons, which leads to temporary or permanent paralysis. In rare cases, respiratory paralysis and death occur.

Individuals that were exposed to PV develop lifelong immunity. The antibodies are present in the tonsils and the gastrointestinal tract. It has to be noted that there is no cross immunity between the three serotypes.

Immunity against all three serotypes can be obtained by vaccination. The Sabin poliovirus vaccine is a live viral vaccine, consisting of one strain of live attenuated PV for each of the three serotypes. The vaccine strains are produced by passage of the virus through monkeys or monkey cells (Sabin, 1965). The attenuation results in a loss of the neurovirulence of the virus in humans while the virus is still able to replicate in the intestine and generate immunity. B- and T- cell response is produced as well as memory B- cells. Vaccine associated disease only occurs rarely (1 in a million doses).

Poliovirus infection was widespread in the 1950's. Large vaccination campaigns helped to eradicate the number of annual diagnosed cases by 99%. However, PV has not been totally eradicated and is still endemic in parts of Nigeria, India, Pakistan and Afghanistan.

1.1.4. Cocksackieviruses:

Cocksackieviruses (CV) belong to the genus of enteroviruses, which means that they replicate in the intestines. They are separated into the Cocksackie A viruses (CVA), consisting of 23 serotypes and Cocksackie B viruses (CVB), consisting of 6 serotypes (Tab.2).

Enterovirus		
Subtype	Species	Serotypes
Human enterovirus A	Cocksackievirus A2- 8, A10, A12, A14, A16	21
	Human enterovirus 71, 76, 89- 92	
	Simian enterovirus 19, 43, 46, A13	
Human enterovirus B	Cocksackievirus B1- 6, A9	57
	Echo- virus 1- 9, 11- 21, 24- 27, 29- 33	
	Human enterovirus 69,73-75,77-88,93,97,98,100,101	
	Simian enterovirus SA5	
Human enterovirus C	Cocksackievirus A1, A11, A13, A17, A19- 22, A24	17
	Poliovirus 1- 3	
	Human enterovirus 95, 96, 99, 102, 104	
Human enterovirus D	Human enterovirus 68, 70, 94	3

Tab.2. Classification of human enteroviruses

The data for this table was taken from [3].

The virus is transmitted via the faecal- oral route from person- to- person for example by unwashed hands or contaminated water. The viruses use receptors that are primarily found in the lymphatic tissue of the throat, where the virus replicates. For CVA various integrin receptors are used, vibronectin for example is the receptor for serotype A9 (Roivainen *et al.*, 1994) (Roivainen *et al.*, 1996), while ICAM-1 is used by A13, A18 and A21 (Colonno, 1986). All six serotypes of CVB use the CAR receptor (*coxsackievirus and adenovirus receptor*) (Bergelson *et al.*, 1997). Additionally DAF (*Decay accelerating factor*) can be used as a receptor by serotype B1, B3 and B5 (Bergelson *et al.*, 1995).

As coxsackieviruses are acid-stable, they can enter the gastrointestinal tract and infect the epithelial layer of the intestine. The incubation period can be between 2 and 35 days, however 1 to 2 weeks are characteristic for most infections. The majority of the infections are asymptomatic or cause only mild symptoms. Neutralizing antibodies are produced. However, in some cases the virus is able to enter the bloodstream and spread to different organs, according to the type of the enterovirus. Severe diseases that are related with coxsackieviruses

include meningitis, encephalitis and myocarditis. A list of the diseases and the serotype of CV that they are related to is given in Tab.3 (King & Mills, 2000; Rieder *et al.*, 2001). CVA is normally related with exanthema, herpangina and hand, foot and mouth disease, whereas CVB is related with internal diseases (Kayser *et al.*, 1998). Coxsackie B4 has been identified as a possible cause of Diabetes mellitus type 1 as CVB4 is able to trigger an autoimmune reaction that results in destruction of the insulin producing beta cells of the pancreas (Roivainen *et al.*, 2000).

	Serotype	disease
Coxsackievirus A	A2- 6, A8, A10	herpangina
	A10	acute lymphatic pharyngitis
	A2, A4, A7, A9, A10, A23	aseptic meningitis
	A7, A9	paralysis
	A2, A4, A6, A16, A23	macular exanthema
	A4- 6, A9, A10, A16	vesicular exanthema
	A5, A10, A16	Hand, foot and mouth disease
	A9, A16	pneumonia
	A21, A24	common cold
	A4, A9	hepatitis
	A18, A20- 22, A24	diarrhoea
	A24	acute haemorrhagic conjunctivitis
Coxsackievirus B	B1- 5	pleurodynia
	B1- 6	aseptic meningitis
	B2- 5	paralysis
	B1- 5	meningoencephalitis
	B1- 5	Bornholm disease
	B1- 5	pericarditis, myocarditis
	B4, B5	Infections of the upper respiratory tract
	B1, B3, B5	macular exanthema
	B5	hepatitis
	B1- 6	fever
	B1, B2, B4	pancreatitis
	B4	Diabetes mellitus Type 1

Tab.3. Diseases related to coxsackieviral infection

The figure was modified according to Roetzer, 2004.

Coxsackie B viruses frequently cause an inflammation of the myocytes of the heart, termed myocarditis. Myocarditis is connected with congestive cardiomyopathy (CCM), a severe disorder of the heart muscle (Liu *et al.*, 1993). In 30% of the patients suffering from CCM, an enteroviral infection of the heart muscle could be detected (Baboonian *et al.*, 1997). CCM may be either acquired or caused by genetic predisposition and causes mainly an enlargement of the ventricles of the heart which results in cardiac insufficiency.

It has been shown that mutations in proteins of the cytoskeleton, especially dystrophin, play an important role in hereditary CCM (Koenig & Kunkel, 1990) (Nigro *et al.*, 1994) (Malhotra *et al.*, 1988). Dystrophin is a cytoplasmic protein and part of the protein complex that

connects the intracellular cytoskeleton of muscle fibres with the surrounding extracellular matrix (Straub *et al.*, 1997) (Chien, 1999).

In CCM that is caused by viral infection, CV 2A^{pro} cleaves the Hinge-3 region of dystrophin, which results in a malfunction of the cytoskeleton (Badorff *et al.*, 1999; Badorff *et al.*, 2000). The same malfunction is found in hereditary CCM, in which the expression of cytoskeleton proteins is affected. The damage of the cytoskeleton by processing of dystrophin is probably used to facilitate the release of viral particles from infected cells (Xiong *et al.*, 2002).

1.2. Translation in eukaryotic cells and during viral infection

Viruses can replicate themselves only when they are infecting a host cell. They are not able to replicate on their own, as their own genome does not code for the machinery for protein synthesis. They are dependent on the host cell for translation. Using the host cell machinery for translation helps the virus to keep its own genome small. Most viruses therefore have found mechanisms to alter the machinery for translation in the host cell in a way that translation of the viral RNA is favoured. In picornaviruses, this results in a complete shut-off of host cell translation, so only viral RNA is translated.

It is necessary to examine the events of translation in eukaryotes first, to understand how picornaviruses achieve the host cell shut-off. Translation is the process in which proteins are produced from an mRNA template, which is read 5' to 3', according to the amino acid sequence which is coded by the mRNA. Usually each mRNA codes for a single protein. As the 4 different nucleotides of the mRNA have to code for the 20 different amino acids that build proteins, a one by one transfer of information is not possible. Therefore, each amino acid of the polypeptide produced is coded by a triplet of bases, the so-called codon.

As these codons are unable to recognise and bind an amino acid directly, an adapter is needed to achieve binding to the amino acid coded for. This adapter is the transfer RNA (tRNA), which ensures that the amino acids are transported to the right codon. It has a 3' terminal site for amino acid attachment and contains base triplets complementary to the codons on the mRNA, the so called anticodons. As there are 61 different base triplets, but only 20 amino acids they code for, the genetic code is degenerate in that several amino acids are encoded by more than one codon. Thus, some tRNAs have to recognize more than one codon. The proposed mechanism for that is the wobble hypothesis, which states that exact base pairing is only necessary for the first two bases, while a mismatch at the last position can be tolerated (Crick, 1966). The adjustment of the tRNA and the codons takes place in the 40S subunit of

the ribosomes, which is the small subunit of the 80S ribosomes of eukaryotes. In the large 60S subunit of the ribosomes the formation of the peptide bond is catalysed, so the polypeptide chain can be elongated.

As long as no protein is synthesized, the two subunits of the ribosome are separated. To start protein synthesis, the 40S subunit scans the mRNA in 5' to 3' direction for an AUG codon and binds to it (Kozak *et al.*, 1978). Then the 60S subunit is recruited and the 80S ribosome complex is composed using a single GTP (Merrick *et al.*, 1979). The mRNA then is drawn through the ribosome. As soon as the codons pass the active site of the ribosome, the nucleotide sequence of the mRNA is translated into an amino acid sequence, using the tRNA as an adapter. When a stop codon is reached, the two subunits of the ribosome separate and the newly synthesized protein is released.

Of the three parts of translation, - initiation, elongation and termination- initiation is the crucial part as most regulatory measures affect this part. Several proteins, the so called eIF's (*eukaryotic initiation factor*) are involved in this process (Schreier *et al.*, 1977; Trachsel *et al.*, 1977). Additionally ATP and GTP are hydrolysed. The eukaryotic initiation factors and their function are described in Tab.4.

Initiation factor	Activity
eIF- 1	facilitates mRNA binding by repositioning of met- tRNA
eIF- 2	formation of the ternary complex
eIF- 2A	AUG- dependent binding of the met- tRNA to the 40S ribosome
eIF- 2B	GTP/GDP exchange during eIF- 2 recycling
eIF- 3	antiassociation of the ribosome subunits, binding to the 40S subunit
PABP	binds to the polyA of the mRNA, provides a link of the poly A tail to eIF- 4G and by this promotes circularization of the mRNA
Mnk1/Mnk2	kinases that phosphorylate eIF- 4E to increase association with the cap- structure
eIF- 4A	ATPase- dependent RNA helicase
eIF- 4E	recognition of the 5' cap, binds to 4G
4E- BP	binds to eIF- 4E in its dephosphorylated form and represses its function, releases eIF- 4E when phosphorylated, which results in increased initiation of translation
eIF- 4G	scaffolding protein, works as adapter and therefore plays a key role in the formation of the mRNA- ribosome translation initiation complex
eIF- 4F	initiation factor complex, composed of eIF- 4A, eIF- 4E, eIF- 4G, PABP and Mnk1 (Mnk2); responsible for the binding of the mRNA to the 40S subunit, the ATPase RNA helicase activity and the interaction between cap structure and polyA tail

Tab.4. Eukaryotic initiation factors and their function

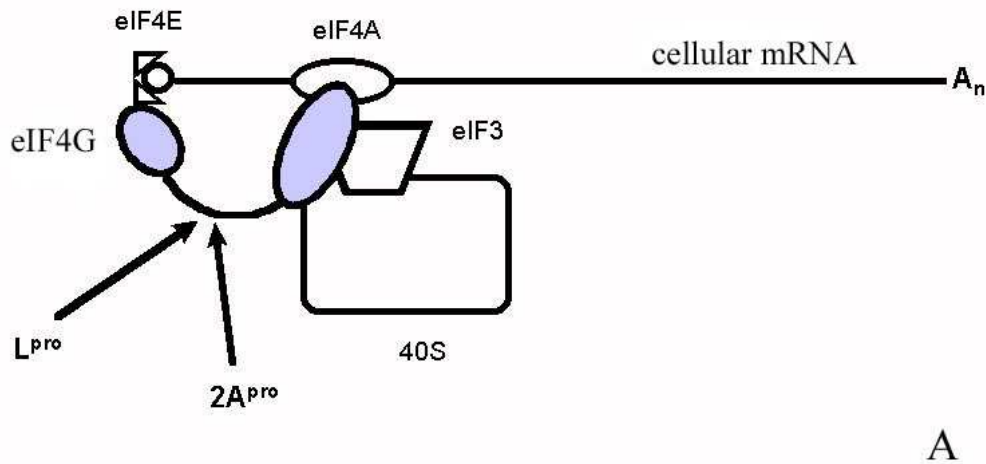
The function of eIF4A, eIF4E, eIF4G and eIF4F is reviewed in (Gingras *et al.*, 1999).

At the start of initiation of translation, a complex composed of the mRNA, the ribosome and the initiator met- tRNA is formed. Initiation of translation can now be performed by two different mechanisms: either cap- dependent or cap- independent via an IRES. In cap- dependent translation initiation, the eIF4E component of the cap binding complex interacts with the 5' cap structure of the eukaryotic mRNA (Shatkin, 1976) and searches for the first start codon, AUG. In cap- independent translation initiation the translation initiation complex is bound to the IRES (*internal ribosome entry site*), a higher ordered RNA structure. The first mechanism is used in eukaryotic cells, whereas picornaviruses use cap- independent initiation as they have an IRES but no cap- structure (Nomoto *et al.*, 1976; Pelletier *et al.*, 1988; Jang *et al.*, 1988). Both ways of translation initiation will now be examined in more detail and are summarized in Fig.4.

In cap- dependent initiation of translation binding to the translation initiation factor complex eIF4F plays an important role. eIF4F is composed of the 3 primary subunits eIF4E, which recognises the 5' cap of the mRNA, eIF4A, a RNA helicase and eIF4G, the adaptor protein plus at least 2 additional factors, PABP and Mnk1. First, cellular mRNA is recognised by the complex and then bound by eIF3. This recruits the 40S ribosomal subunit in a complex with eIF2- Met- tRNA- GTP, eIF3 and eIF4A to the mRNA. Then the cellular mRNA is unwound by eIF4A close to the AUG, where the ribosome can bind. After eIF4E binds to the cap, the N-Terminus of eIF4G binds eIF4E, while the C-Terminus binds to eIF4A. The 40S subunit of the ribosome binds to eIF4G indirectly via eIF3. eIF4G has a crucial function in translation as it connects the initiation factor complex and the 40S subunit of the ribosome.

The 40S subunit then scans the 5' noncoding region of the mRNA until the authentic start codon is reached. At the AUG the 60S subunit of the ribosome joins the complex and the initiation factor complex is released. Following this, elongation starts and the appropriate amino acids are guided to the exit-site of the ribosome. The polypeptide chain is elongated until a stop- codon is reached. There the release protein eRF1 exerts hydrolysis between the polypeptide chain and the tRNA. Finally the ribosome releases the protein and the translation complex falls apart.

Cap dependent initiation of translation



Cap independent initiation of translation

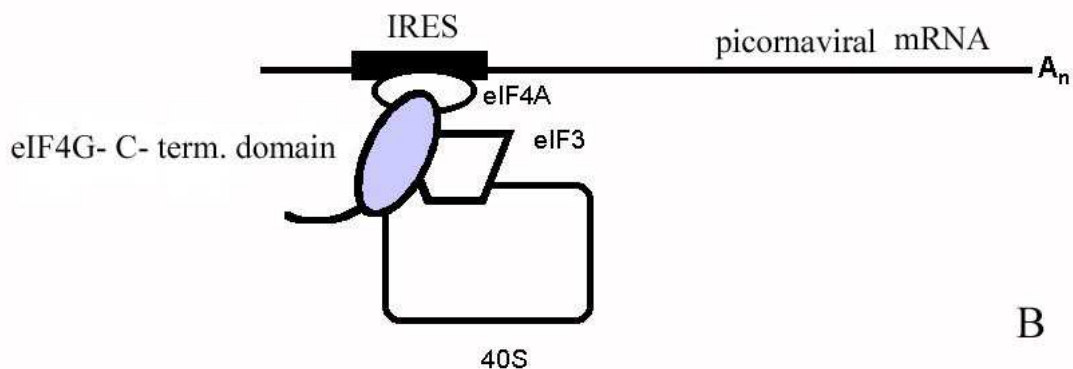


Fig. 4: Cap dependent and cap independent initiation of translation

In picture A the formation of the translation initiation complex during cap dependent translation is shown. Here eIF4E binds to the 5' cap of cellular mRNA and is bound to the N-terminus of eIF4G. The C-terminus of eIF4G binds the 40S ribosomal subunit via eIF3 and eIF4A directly thereby connecting the cap with the 40S. The arrows indicate where L^{pro} and 2A^{pro} cleave eIF4G. This cleavage results in the separation of the cap bound to eIF4E from the rest of the initiation complex, which disables protein synthesis from cellular mRNA and results in the shut-off of host cell translation. Picture B shows the situation after the cleavage for cap independent translation. The C-terminal domain of eIF4G is still able to form a complex with picornaviral RNA, as their 5' noncoding structure contains an IRES, which can be used instead of a cap structure. Figure 4 was taken from (Seipelt *et al.*, 1999)

Picornaviruses are not able to use the cap-dependent mechanism for initiation of translation as their RNA genome does not contain a 7- methyl G cap structure as it is found at the 5' end of cellular mRNA. Instead of the cap structure a small viral protein, VPg, is linked to their 5' end (Lee *et al.*, 1977; Flanagan *et al.*, 1977). Additionally picornaviruses have a long, highly

structured 5' noncoding region and several non- authentic start codons that could preclude ribosomal scanning (Kitamura *et al.*, 1981; Racaniello *et al.*, 1981). Therefore, initiation of translation in picornaviruses is performed by a cap-independent mechanism known as internal ribosome entry (Pelletier *et al.*, 1988; Jang *et al.*, 1988). In cap- independent translation the 40S ribosomal subunit binds to an internal ribosome entry site (IRES) instead of the cap and initiation starts at the authentic start codon.

Internal ribosome entry sites have been identified for all picornaviruses and are located in the 5' noncoding region, a part of the genome which sequence and RNA secondary structure are highly conserved among the viral serotypes of each genera. The picornaviral IRESs are mainly divided into two groups according to their structure homology and sequence similarity (Wimmer *et al.*, 1993). Group 1 includes the IRESs of enteroviruses and human rhinoviruses, group 2 that of cardio- and aphthoviruses. In entero- and rhinoviruses the 5' noncoding region contains six major stem-loops upstream of the start site of translation, the IRES being located between stem-loop II and V (Pelletier *et al.*, 1988). Group 2 IRESs as well contain highly conserved stem-loop structures, the largest loop being stem-loop I.

The use of an IRES that enables cap-independent initiation of translation is highly favourable for picornaviruses as it allows them to interfere with host cell translation. Picornaviruses evolved a mechanism that is used to inhibit protein synthesis of the host cell that uses cap-dependent initiation of translation. This finally results in the host cell shut-off (Kräusslich *et al.*, 1987; Lloyd *et al.*, 1987; Jewell *et al.*, 1990). This is achieved by the cleavage of the two isoforms of eIF4G. eIF4G binds eIF3 that is associated with the ribosome and delivers the 40S subunit to the mRNA in doing so (Lamphear *et al.*, 1995; Mader *et al.*, 1995). It consists of two isoforms: eIF4GI and eIF4GII (Yan *et al.*, 1992; Gradi *et al.*, 1998) which are 41% identical and functionally complement each other (Gradi *et al.*, 1998; Imataka *et al.*, 1998).

As remarked above, eIF4G has a crucial function in translation as it connects the initiation factor complex and the 40S subunit of the ribosome. Its N-terminus binds directly to eIF4E, which interacts with the cap structure of the mRNA, while its C-terminus binds to eIF4A and the small ribosomal subunit via eIF3. After the cleavage of the two isoforms of eIF4G by the picornaviral proteases, it can not bind to eIF4E anymore, while the 40S subunit and eIF4A can still be bound on the C- terminus. This separation of the cap structure bound to eIF4E from the 40S subunit means that initiation of translation via an IRES is still possible, whereas cap- dependent translation is cut off. In cap- independent initiation of translation, only the C-terminal end of eIF4G is used, as the internal IRES can be used for initiation of translation. As cellular mRNAs are translated in a cap- dependent manner, translation in the host cell

stops, whilst the viral mRNA can still be translated. It has been shown in PV1 and HRV14 that host cell shut off coincides with the cleavage of eIF4GII while the cleavage of eIF4GI occurs earlier (Gradi *et al.*, 1998; Svitkin *et al.*, 1999), whereas in HRV2 both isoforms are cleaved at similar rates (Seipelt *et al.*, 2000).

The cleavage of the two isoforms of eIF4G is performed by the picornaviral proteases 2A^{pro} or L^{pro}. In enteroviruses and human rhinoviruses the cleavage is exerted by 2A^{pro}, in FMDV by L^{pro}. A deletion of the proteinase responsible for the cleavage of eIF4G results in an attenuated phenotype (Kanno *et al.*, 2001; Sakoda *et al.*, 2001; Brown *et al.*, 1996). The cleavage site on eIF4GI of HRV2, PV1 and CVB4 2A^{pro} has been identified as LeuSerThrArg⁶⁸¹*GlyProProArg (Lamphear *et al.*, 1993; Zamora *et al.*, 2002), that of eIF4GII lies seven amino acids C-terminal of it being ProLeuLeuAsnVal⁶⁹⁹*GlySerArgArg (Gradi *et al.*, 2003). The cleavage site for HRV14 2A^{pro} has not yet been determined. L^{pro} cleaves eIF4GI seven residues apart from rhinovirus 2A^{pro} at AlaAsnLeuGly⁶⁷⁶*ArgThrThrLeu (Kirchweger *et al.*, 1994) and eIF4GII immediately adjacent to the site cleaved by HRV 2A^{pro} at LeuAsnValGly⁷⁰⁰*SerArgArgSer (Gradi *et al.*, 2004). Further information on the activity of these two proteases will be given in the following chapter.

1.3. Picornaviral proteases

1.3.1. General information

Proteases are enzymes that are able to cleave proteins or peptides by hydrolysis of peptide bonds. They can be qualified either by the type of proteolytic reaction they catalyse or by their active site. Proteases which cut at the N-Terminus are classified as amino peptidases, the ones that cut at the C-Terminus are regarded as carboxy peptidases. According to the reactive groups in their active site, they can be divided into six groups: serine proteases (e.g. chymotrypsin), threonine proteases (e.g. the proteasome), cysteine proteases (e.g. papain), aspartic acid proteases (e.g. pepsin), glutamic acid proteases (e.g. the germination protease) and metalloproteases (e.g. pepsin) (Barrett *et al.*, 1998).

Hydrolysis of the peptide bond is achieved by different mechanisms. One way is to create a nucleophilic cysteine or threonine residue or make a water molecule nucleophilic, so it can attack the carbonyl group of the peptide bond. A frequently used strategy to create a nucleophile is using a catalytic triad, a set of three functional groups in the active site of proteases. These functional groups typically are a nucleophilic which attacks the carbonyl group of the peptide bond; an acid- base catalyst which protonates the leaving group and the oxyanion hole; and an electrophilic structure that stabilizes the negatively charged carbonyl group (Ryan & Flint, 1997; Chernaiia *et al.*, 1993). Usually a histidine is used to activate serine, cysteine or threonine as a nucleophile.

Proteases play a major role in picornaviruses as their genome was shown to encode proteolytic enzymes that are responsible for the processing of the viral polyprotein into the mature viral proteins (Lawrence & Thatch, 1975; Pelham, 1987; Gorbalenya *et al.*, 1979; Palmenberg *et al.*, 1979; Svitkin *et al.*, 1979). The first proteinase that was found was 3C^{pro}, which is responsible for the majority of cleavage events on the polyprotein. Following this, the 2A^{pro} of entero- and rhinoviruses (Toyoda *et al.*, 1986) and the L^{pro} of FMDV (Strebel & Beck, 1986) were identified.

Alignments of the amino acid sequences of picornaviral proteases with well-established models of these protease classes can be used to predict their structure. For the picornaviral 2A and 3C proteases this revealed that they are serine proteases with a chymotrypsin- like fold and a cysteine in their active site, whereas Leader protease is a cysteine protease with a

papain- like fold (Guarné *et al.*, 1998; Petersen *et al.*, 1999). The sequences of picornaviral proteases have been reviewed (Ryan & Flint, 1997).

The RNA genome of picornaviruses is translated into a single polyprotein, which is subsequently cleaved into the various viral proteins by the picornaviral proteases. The first cleavage is used to separate the structural proteins at the N-Terminus from the non-structural proteins at the C-Terminus (Toyoda *et al.*, 1986). This separates P1, the precursor of the capsid proteins VP1 to VP4, from P2 and P3, the precursor of the non-structural proteins 2A to 2C and 3A to 3D. Following this, P1 is cleaved into VP1, VP3 and VP0. VP0 is the precursor protein of VP2 and VP4. P2 and P3 are separated and then cleaved into the according proteins.

The first cleavage performed by the picornaviral proteases is a *cis* cleavage used to free themselves from the polyprotein (Toyoda *et al.*, 1986). As the proteases perform the *cis* cleavage cotranslationally, the full length polyprotein is never found in living cells. As soon as the proteases have cleaved themselves from the polyprotein, they start to cleave in *trans*. This strategy is used to control the genetic expression of the polyprotein. Proteins with different activities can be generated using different cleavage sites.

The first cleavage is achieved by 2A^{pro} in rhino-, entero-, erbo-, cardio-, tescho- and aphthoviruses. In hepato- parecho- and kobuviruses, 2A is not proteolytically active and the cleavage between structural and non- structural proteins is performed by 3C^{pro} (Jia *et al.*, 1991; Martin *et al.*, 1995; Schultheiss *et al.*, 1995). In cardio- and aphthoviruses, the leader protein precedes P1. In aphthoviruses the leader protein is a papain-like cysteine protease (Gorbalenya *et al.*, 1991; Guarné *et al.*, 19989) which cleaves between its own C-Terminus and the N-Terminus of VP4. In cardioviruses the leader protein is not proteolytically active, it abrogates the anti- viral response of the host by interfering with nucleo-cytoplasmic transport (Delhaye *et al.*, 2004; Lidsky *et al.*, 2006; Porter *et al.*, 2006). The cleavage between the C-terminus of the leader protein and the N-terminus of VP4 is achieved by 3C^{pro}. The leader protein in cardioviruses abrogates host anti-viral response by interfering with nucleo-cytoplasmic transport. All further cleavages except the cleavage of VP0 to VP2 and VP4 are performed by 3C^{pro} or its precursor 3CD^{pro}, which are proteolytically active in all picornaviruses. The cleavage of VP0 to VP2 and VP4 takes place during maturation of the viral particles after VP0 has been assembled into the procapsid and is catalysed by a yet unknown proteolytical activity (Arnold *et al.*, 1987; Harber *et al.*, 1991; Basavappa *et al.*,

1994). A scheme of the processing of the polyprotein is given in Fig.5, Fig.6 summarises the proteolytic processing in the picornavirus genera.

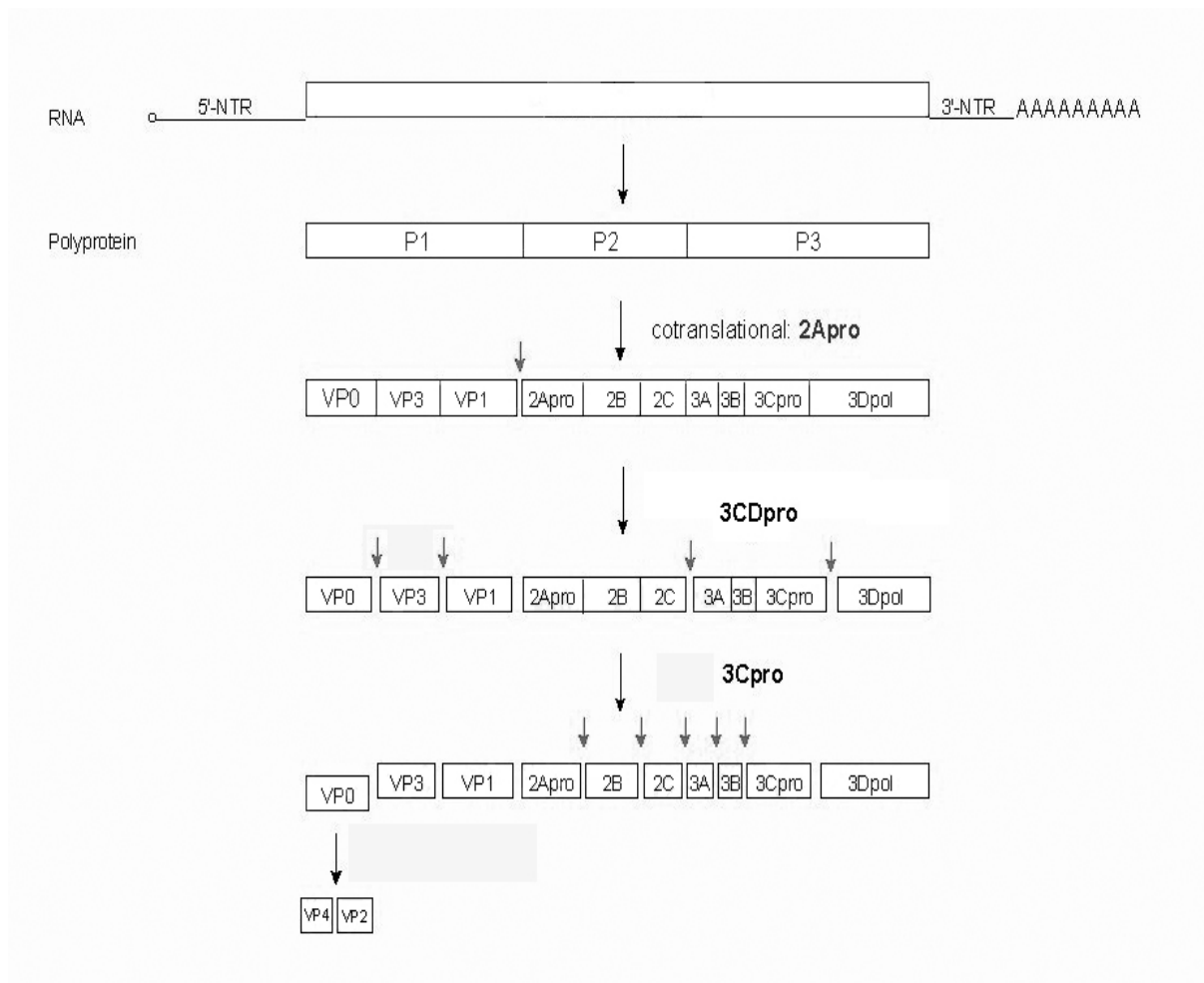


Fig.5: Polyprotein processing

A summary of the steps for polyprotein processing in rhino- and enteroviruses is given in this figure. The first cleavage is performed by the 2A^{pro} which separates structural from non-structural proteins. All further cleavages are performed by 3C^{pro} or its precursor 3CD. The figure above was modified according to [4].

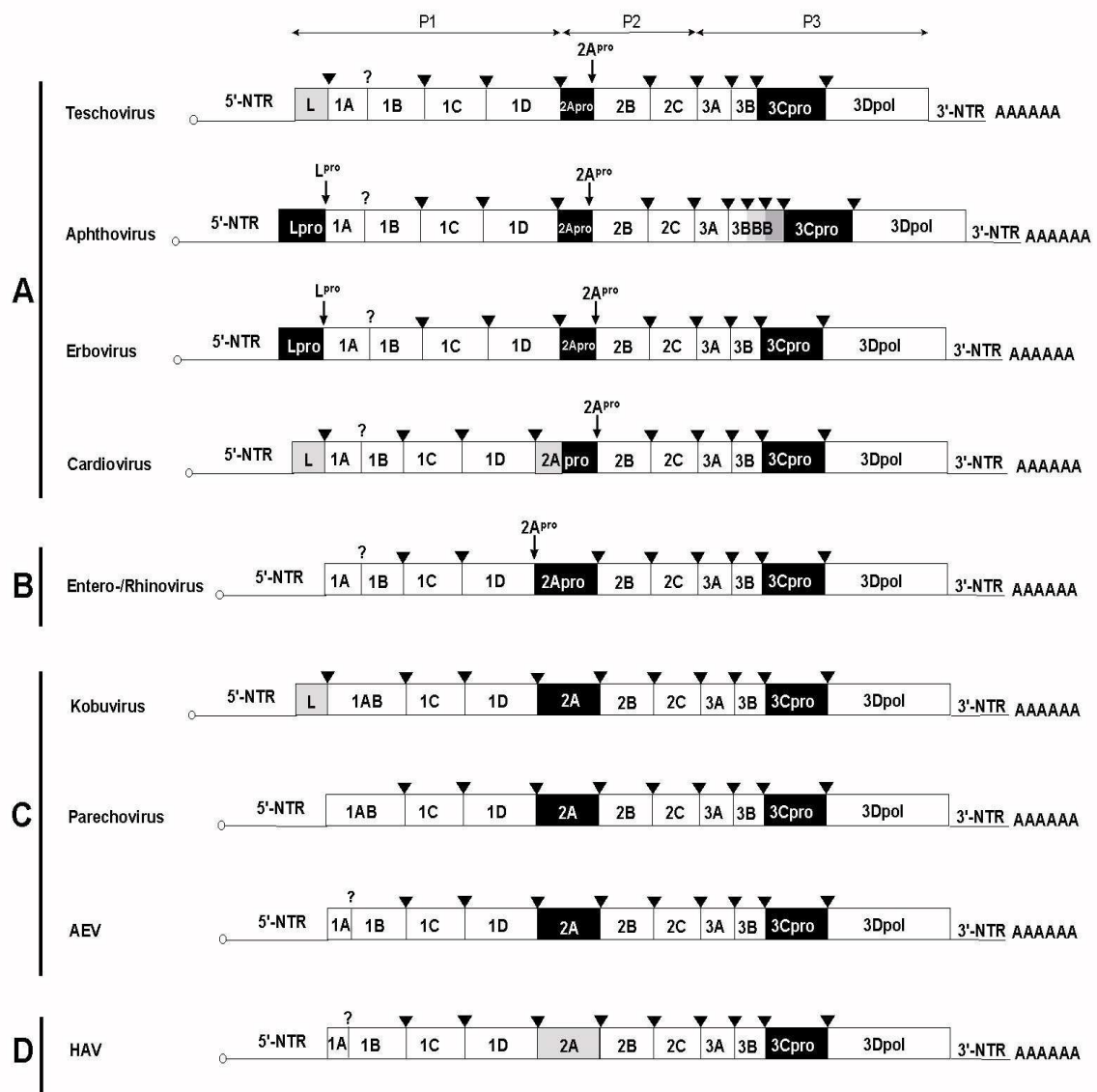


Fig.6: Processing in the different genera

The figure shows the processing of the polyprotein in the different picornaviral genera and the involved proteases. The figure was taken from [5].

1.3.2. Leader protease (L^{pro})

The leader protein is present in cardioviruses and aphthoviruses, however, it is only proteolytically active in aphthoviruses. The L^{pro} of aphthoviruses such as FMDV is a cysteine protease with a papain- like fold. L^{pro} is the first protein encoded on the FMDV polyprotein and first cuts between its own C-Terminus and the N-Terminus of VP4 to free itself from the polyprotein (Strebel & Beck, 1986). Additionally, it cleaves the two isoforms of eIF4G inhibiting the cap- dependent translation of the host cell. This cleavage results in a shut-off of host cell translation (Devaney *et al.*, 1988). $2A^{\text{pro}}$ performs a similar cleavage in rhino- and enteroviruses, but has been shown to cleave at a different sequence (Kirchweiger *et al.*, 1994). The catalytical triad of L^{pro} consists of cysteine, histidine and aspartic acid (Roberts & Belsham, 1995; Skern *et al.*, 1998). The crystal structure of foot- and- mouth- disease L^{pro} has been published (Guarnè *et al.*, 1998). It reveals a globular catalytic domain very similar to that of the papain superfamily and a flexible C-terminal extension. This extension supports intramolecular self-processing and the binding of eIF4GI (Foeger *et al.*, 2002).

1.3.3. 3C protease ($3C^{\text{pro}}$)

The 3C protease and its precursor $3CD^{\text{pro}}$ are responsible for the majority of cleavage reactions during polyprotein processing and are found in all genera of picornaviruses (Dougherty & Semler, 1993). According to inhibitor studies, it was predicted to be a cysteine protease, however three dimensional modelling and sequence alignments implied a chymotrypsin-like fold that is normally typical for serine proteases. These predictions were verified by the crystal structures of the $3C^{\text{pro}}$ of hepatitis A virus (Allaire *et al.*, 1994), HRV14 (Matthews *et al.*, 1994), PV1 (Mosimann *et al.*, 1997) and FMDV (Birtley *et al.*, 2005). It has to be remarked that the $3C^{\text{pro}}$ has α - helices on the C- and the N-termini, whereas chymotrypsin has an α - helix only at the C-Terminus. These helices play a role in RNA binding, which takes place at the 5' noncoding region of the picornaviral genome. The 5' terminal 100 nucleotides of entero-, rhino-, and hepatoviruses form a cloverleaf structure that is required for viral replication. However, the cloverleaf structure has no binding site for the RNA-dependent RNA polymerase ($3D^{\text{pol}}$). Instead this structure binds to $3C^{\text{pro}}$ or its precursor $3CD^{\text{pol}}$ (Andino *et al.*, 1990), binding to the precursor was reported to be 10-fold stronger (Andino *et al.*, 1993). The RNA binding site of $3C^{\text{pro}}$ comprises one face each of the N- and C-terminal helices as well as the part of the polypeptide chain that links the two domains. It was shown that the amino acid sequence Lys Phe Arg Asp Ile is involved in RNA binding.

This sequence is conserved in most picornaviruses, which implies that RNA binding has tight evolutionary constraints (Matthews *et al.*, 1994; Bergmann *et al.*, 1997; Bergmann & James, 1999).

On the opposite face of the molecule to the RNA binding site the active proteolytic site is located. In HAV 3C^{pro} the catalytical triad is composed of cysteine, histidine and aspartic acid - however the side chain of aspartic acid does not seem to take part in catalysis. In poliovirus, aspartic acid is substituted by glutamic acid, which does take part in catalysis. Although 3C^{pro} is a cysteine protease, this composition of the catalytic triad resembles the enzymes the serine proteases of the chymotrypsin family, where a carboxyl group is found in the catalytic triad as well. In the cysteine protease papain asparagines is found as third member of the triad. Therefore it was discussed whether the reactive nucleophile of the 3C^{pro} is the thiolate ion of the thiolate- imidazolium ion pair as in papain (Polgar, 1974 and 1989) or the reaction of the nondissociated thiol group is catalysed by general base catalysis, a mechanism used in serine proteases, where the active site OH group attacks the carbonyl carbon atom of the substrate. It was shown that the 3C^{pro} of poliovirus does not have a catalytically active thiolate-imidazolium ion pair (Sárkány *et al.*, 2001). Therefore the imidazole assistance in hydrolysis is very likely general base catalysis as in serine proteases. This is followed by the nucleophilic attack on the carbonyl group of the peptide backbone, which frees the amino terminal of the backbone. An acyl- enzyme intermediate is formed, then the enzyme is deacylated by a water molecule and the carboxy terminal part of the protein is released. The negatively charged oxygen of the substrate is stabilized in the oxyanion hole during processing.

1.3.4. 2A protease (2A^{pro})

The picornaviral 2A protease is a multifunctional enzyme. In rhino- and enteroviruses the 2A^{pro} performs the first cleavage on the polyprotein by cleaving between its own N-Terminus and the C-Terminus of VP1, thereby separating the capsid protein precursor from the non-structural proteins (Toyoda *et al.*, 1986). Additionally, 2A^{pro} cleaves the two isoforms of eIF4G in *trans*, which leads to the shut-off of host cell translation. The IRES activity is maintained or even enhanced after the cleavage of eIF4G by 2A^{pro} (Hambidge & Sarnow, 1992; Liebig *et al.*, 1993; Ziegler *et al.*, 1995; Borman *et al.*, 1997). Furthermore, 2A^{pro} is involved in the cleavage of poly- A- binding- protein (PABP) (Joachims *et al.*, 1999; Kerekatte *et al.*, 1999). The interaction of PABP with eIF4G also stimulates the IRES-dependent translation (Sachs *et al.*, 1997; Svitkin *et al.*, 2001). This is important in order to

achieve sufficient production of viral proteins as the IRES in entero- and rhinoviruses is quite weak compared to other picornaviruses (Bormann *et al.*, 1995)). In addition to protein processing 2A^{pro} may also play a role in RNA replication (Molla *et al.*, 1993; Yu *et al.*, 1995).

Like 3C^{pro} the 2A protease was predicted to have a chymotrypsin-like fold by alignment to known structures, although they have a cysteine as their active site nucleophile instead of the serine of chymotrypsin (Bazan & Fletterick, 1988; Gorbalenya *et al.*, 1989). The crystal structure of HRV2 2A^{pro} confirmed these predictions (Petersen *et al.*, 1999) and is shown in Figure 7. This revealed more details on the mechanism of both intra- and intermolecular cleavage and substrate specificity. The structure and function of the 2A^{pro} will now be examined in detail.

As mentioned above the HRV2 2A^{pro} is a cysteine protease with a chymotrypsin-like fold. Its overall structure resembles that of *Streptomyces griseus* proteinase B (SGPB) (Read *et al.*, 1983; Petersen *et al.*, 1999) with the 3C^{pro} of PV1. Similarly, it comprises two subdomains built up by β -strands that are connected by a so called interdomain loop. However, while chymotrypsin-like proteases usually have eight β -strands in the N-terminal subdomain, HRV2 2A^{pro} has only four. Another unusual feature of the HRV2 2A^{pro} is the presence of a tightly bound zinc ion coordinated at the beginning of the second subdomain on the opposite face of the molecule to the active site by three cysteine and one histidine residue (Cys52, Cys54, Cys112 and His114) at the beginning of the second subdomain on the opposite face of the molecule to the active site (Sommergruber *et al.*, 1994; Sommergruber *et al.*, 1997; Voss *et al.*, 1995). As this zinc ion is not accessible for ligands according to inhibitor studies and more than 20Å away from the active site it does not seem to be involved in catalysis, but increases the stability of the protein. It seems likely that it maintains stability by compensating for the four missing β -strands of the N-terminal domain and takes over the role of a disulfide bridge found at an equivalent position in extracellular chymotrypsin-like proteases. Normally disulfide bridges are not formed inside cells due to the reducing environment, so HRV2 2A^{pro} may have evolved the zinc binding site as an alternative to a disulfide bridge in order to achieve more stability (Petersen *et al.*, 1999; Tsukada *et al.*, 1985; Glaser *et al.*, 2003).

The catalytical triad of 2A protease is located in the cleft between the N-terminal and the C-terminal domain and is composed of histidine as acid-base-catalyst, cysteine as a nucleophile and aspartic acid. In HRV2 2A^{pro} the position and orientation of the active site residues are very similar to that of SGPB, being His18, Asp35 and Cys106 in HRV2 2A^{pro} and His57,

Asp102 and Ser195 in SGPB. In HRV2 2A^{pro} the His18 is stabilized by a net of hydrogen bonds in which the Asp35 is involved (Matthews *et al.*, 1994).

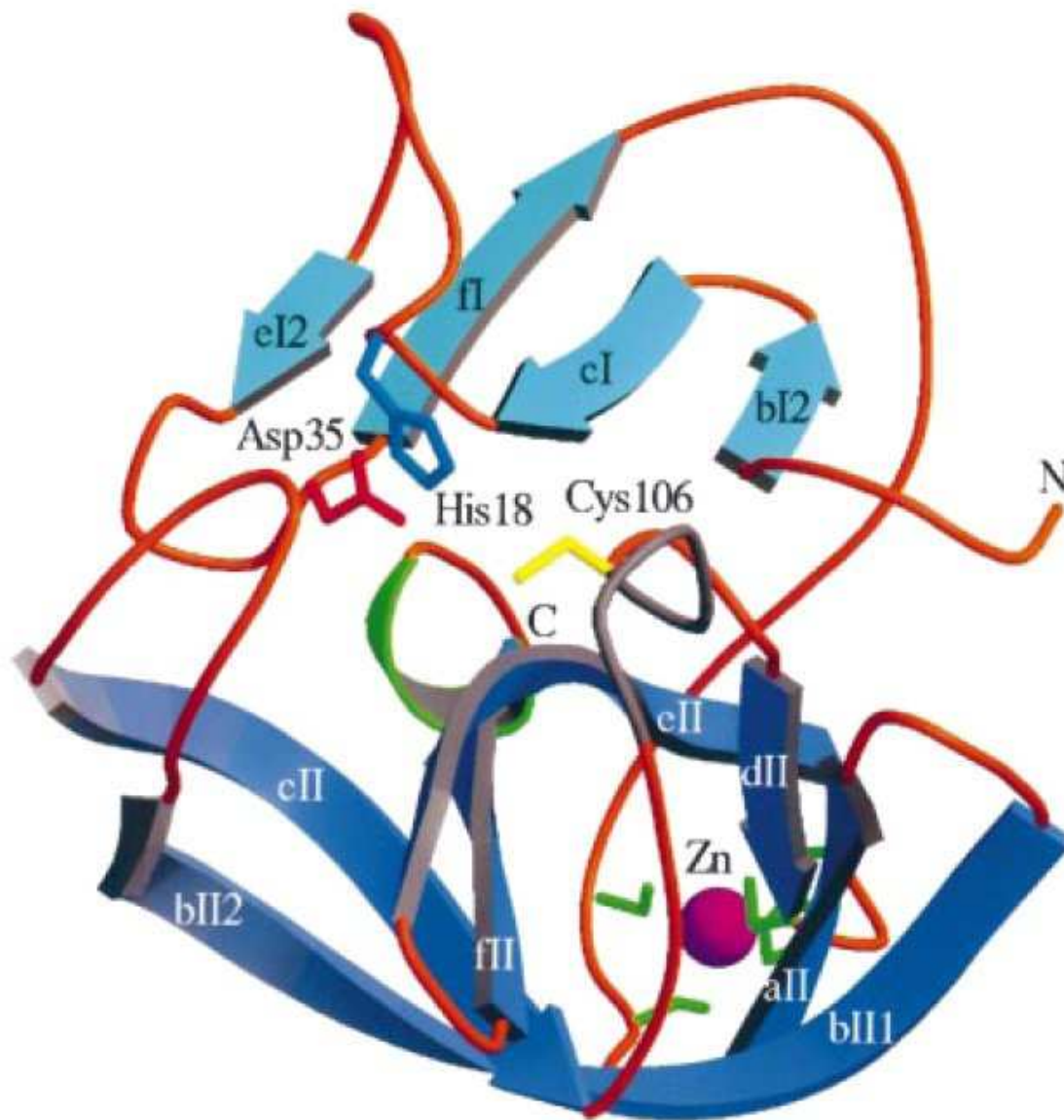


Fig. 7: Crystal structure of HRV2 2A^{pro}

Ribbon diagram of the overall structure of HRV2 2A^{pro} protease that also shows the members of the catalytic triad and the coordinated zinc ion. The Figure has been taken from Petersen *et al.*, 1999.

The 2A proteinases of rhinoviruses and enteroviruses perform the first cleavage of the polyprotein by cleaving in *cis* between their own N-Terminus and the C-Terminus of VP1. This results in the separation of structural proteins from non-structural proteins (Toyoda *et al.*, 1986). This initial cleavage event occurs co-translationally, which explains the absence of full-length viral polyprotein in infected cells.

Secondly, the 2A^{pro} cleaves the two isoforms of eIF4G in *trans*. This cleavage separates the N-terminal eIF4E binding domain and the C-terminus eIF3 binding domain of eIF4G

(Lamphear *et al.*, 1995), thereby precluding the recruitment of capped mRNA to the ribosomes (Bormann *et al.*, 1997; Liebig *et al.*, 1993). This leads to a shut-off of the cap-dependent translation of the host cell (Kräusslich *et al.*, 1987). The translation of viral proteins is not disrupted by that as the 5' UTR of picornaviruses includes an IRES that enables cap- independent translation (Jackson *et al.*, 1990; Jang *et al.*, 1990; Belsham & Sonenberg, 1996). It was even shown that IRES-dependent initiation of translation is stimulated by 2A^{pro} cleavage of eIF4G (Hambidge & Sarnow, 1992; Liebig *et al.*, 1993; Ziegler *et al.*, 1995; Bormann *et al.*, 1997).

HRV2 2A^{pro} initially recognises and binds eIF4GI away from the scissile bond between amino acids 600- 674, which are located immediately N-terminal to the amino acids where cleavage occurs, but do not include them. Mutagenesis and binding studies located the eIF4G binding domains away from the substrate binding cleft, thus representing exosites (Foeger *et al.*, 2002, 2003). More work on the processing of eIF4G revealed that eIF4G cleavage is more efficient in presence of eIF4E (Haghighat *et al.*, 1996; Ventoso *et al.*, 1998) and *in vivo* studies gave evidence that the two isoforms of eIF4G are not cleaved simultaneously in all picornaviruses. During HRV2 infection both isoforms are cleaved after 3 hours, which correlates with the host cell shut-off of infected cells (Seipelt *et al.*, 2000). In HRV14, on the other hand, eIF4GI is processed after less than 3 hours, while eIF4GII is processed after 5 hours (Gradi *et al.*, 2003). The host cell shut-off, which is never completed in HRV14, correlates with the cleavage of eIF4GII.

As self-processing and eIF4G cleavage are performed by all rhino- and enteroviruses, a similar substrate specificity might be anticipated. However, the sequences of HRV2 and HRV14 2A proteinases show only 40% amino acid identity (Wang *et al.*, 1998), and alignments of the amino acid sequences indicate that HRV14 2A^{pro} is more closely related to polioviral or coxsackieviral 2A proteinases. The NMR structure of CVB4 2A^{pro} in terms of overall fold, it is similar to the crystal structure of the HRV2 2A^{pro}, but the low level (40%) of sequence identity leads to a substantially different surface (Baxter *et al.*, 2006). In the various rhino- and enteroviruses, the cleavage site between VP1 and 2A^{pro} shows a large degree of variation in its amino acid sequence. The amino acid sequences that are recognised by the 2A^{pro} in the different cleavage reactions are given in table 5 (cleavage sites are numbered according to the nomenclature of Berger & Schechter, 1970).

	P5	P4	P3	P2	P1	*	P1'	P2'	P3'	P4'
HRV2	I	I	T	T	A	*	G	P	S	D
HRV14	D	I	K	S	Y	*	G	L	G	P
PV1	D	L	T	T	Y	*	G	F	G	H
CVB4	S	L	I	T	T	*	G	P	Y	G
eIF4GI	T	L	S	T	R	*	G	P	P	R
eIF4GII	P	L	L	N	V	*	G	S	R	R

Tab.5: Amino acid sequences at the cleavage sites of rhino- and enteroviruses recognised by 2A^{pro}: The sequences were taken from the following sources: HRV2 (Skern *et al.*, 1985); HRV14 (Stanway *et al.*, 1984); PV1 (Kitamura *et al.*, 1981); CVB4 (Jenkins *et al.*, 1987); eIF4GI (Lamphear *et al.*, 1995); eIF4GII (Gradi *et al.*, 1998). The cleavage site is marked by the star, the colour code is explained in the text.

As it was mentioned above, the cleavage sites recognised by rhino- and enteroviral 2A proteinases are rather heterogeneous. However, the P1' site of all rhino- and enteroviruses is highly conserved. At this position, marked in red in the table above, a glycine is found in all serotypes. Mutations in which the P1' glycine is replaced by another amino acid are deleterious in all cases (Glaser & Skern, 2000).

At the P2' positions a proline is found in all rhinoviruses except HRV14 and CVB4. Deletion of this proline has been shown to eliminate cleavage in HRV2. The crystal structure of HRV2 2A^{pro} shows that this residue sticks out of the active site (Petersen *et al.*, 1999). In eIF4GII, a serine is found at P2', which is a residue with a short side chain that should be also recognised by HRV2 2A^{pro}. This might explain why HRV2 2A^{pro} recognises both eIF4GI and eIF4GII equally well (Seipelt *et al.*, 2000). In HRV14 and PV1, the P2' position is occupied by leucine and phenylalanine respectively; both large hydrophobic residues. The hydrophilic serine at the eIF4GII P2' seems to inhibit substrate recognition by 2A^{pro}. It has been shown that a peptide that mimics HRV14 recognises HRV14 2A^{pro} 12 times better when the leucine at P2' is substituted with proline (Wang *et al.*, 1998b). However, this is only true in *trans* cleavage. No difference can be seen in the *cis* cleavage. Additionally, it has to be noted that HRV14 2A^{pro} does not recognise the eIF4GI sequence in *cis*, although it has a proline at P2'. This indicates different mechanisms for *cis* and *trans* cleavage. In P3' and P4', a large variety of amino acids can be tolerated.

At the P1 site, alanine and valine are preferred by most rhinoviruses and enteroviruses. However, in HRV14 and polioviruses, a tyrosine is found at P1 (marked in green); in coxsackie viruses B1- B4, there is a threonine, both hydrophilic residues. Recent data indicates that the tyrosine at P1 plays an important role in substrate recognition in HRV14. It has been shown that HRV14 2A^{pro} could not carry out self-processing when the cleavage site was replaced by that of eIF4GI. However, replacing of the arginine at P1 of eIF4GI by

tyrosine was sufficient to restore the self-processing activity. The substitution of tyrosine by arginine in the wild type cleavage site of HRV14 on the other hand eliminated cleavage (Sousa *et al.*, 2006).

At the P2 threonine or asparagine are found in all rhino- and enteroviruses, as well as in eIF4GI and eIF4GII, with the exception of HRV14, where serine occupies the P2 position. At P3 threonine or lysine are found, only in CVB4 isoleucine is found at P3. The P4 residue is aliphatic in all rhino- and enteroviruses. In the sequences used in experiments for this thesis, they are always leucine or isoleucine (marked in blue). This indicates that a hydrophobic residue at P4 is important in substrate recognition by 2A^{pro}.

The crystal structure of HRV2 2A^{pro} and the solution structure of CVB4 2A^{pro} (Baxter *et al.*, 2006) facilitated understanding of the substrate recognition of 2A^{pro}. It has to be remarked that given the low level of identity between the different entero- and rhinoviruses, more residues involved in substrate recognition may only be recognised when the crystal structures of HRV14 2A^{pro} and other picornaviral 2A proteinases become available.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Plasmids

For all *in vitro* experiments pCITE- A1 (® Novagen) was been used as vector (Fig.8). pCITE- A1 includes a CITE- sequence (*Cap Independent Transcription Enhancer*), that derives from encephalomyocarditis virus (EMCV) and facilitates internal initiation of protein synthesis. The 3705bp vector codes for a gene that gives resistance to ampicillin. It contains the origin of replication of pUC that leads to high yields of plasmid, the promoters T7 and SP6. The viral sequences were cloned in the plasmid downstream of the EMCV internal ribosome entry site.

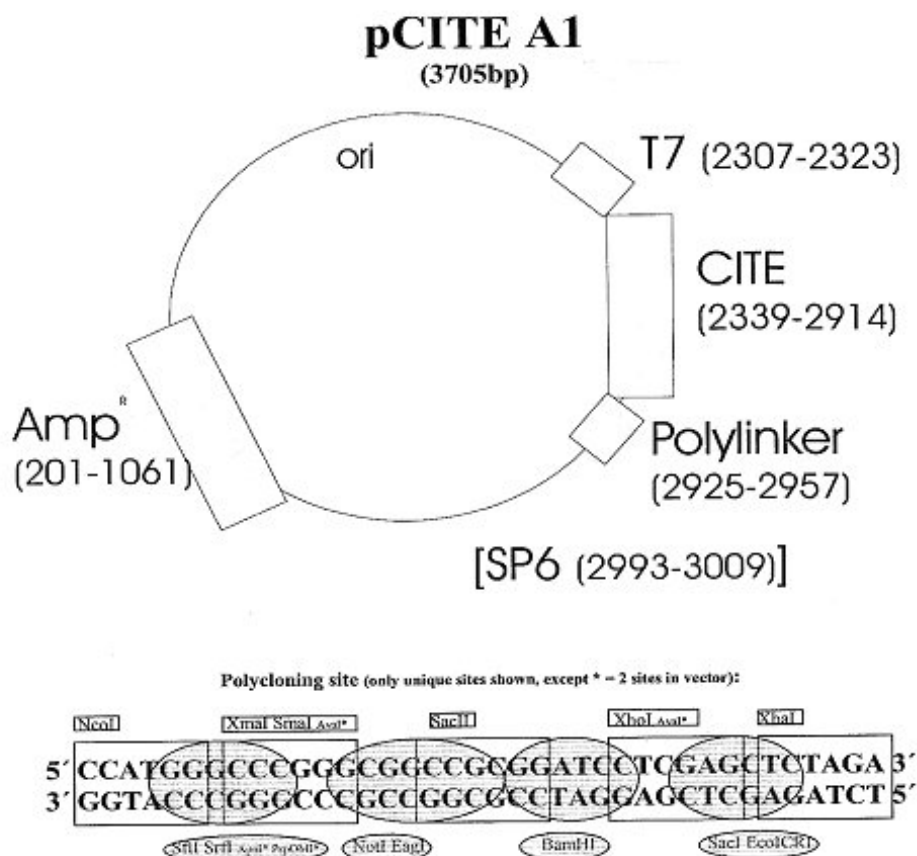


Fig.8: pCITE A1 vector

For HRV2 and HRV14 the EMCV insert was removed and the DNA-fragments of VP1- 2A^{pro} were introduced between the *NcoI* (2925bp) and *BamHI* restriction sites. This *BamHI* restriction site was not present in the original Novagen pCITE- 1 (Glaser and Skern, 2000).

2.1.2. Bacterial strains

For all cloning experiments the bacterial strain *E.coli* TOP10F' was used.

2.1.3. Media

Luria- Bertani (LB) medium:

- 5 g/l yeast extract (Gibco BRL)
- 10g/l Pepton (Gibco BRL)
- 10g/l NaCl (Fluka)
- pH 7.5 (adjusted with NaOH)

agarose plates:

- LB medium
- 1.5 % (w/v) agarose

antibiotics:

ampicillin (1000x stock in H₂O; 100mg/ml)

2.1.4. Oligonucleotides

In the table 6 all oligonucleotides that have been used are displayed with their sequence and function.

Primer	Nucleotide Sequence	Function
TIM 1401	5' AATTGTGACCAGACCAATTATCACTACAA TGGGCCCCAGTGACATGTATGTTTCATGTAG 3'	Creation of the mutant P1M in HRV2VP12A (fwd)
TIM 1402	5' GTTACCTACATGAACATACATGTCACTGGGGC CCATTGTAGTGATAATTGGTCTGGTCAC 3'	Creation of the mutant P1M in HRV2VP12A (rev)
TIM 1403	5' CCGGTAATTAAGAAGAGGAAAGGTGACA TTAAATCCATGGGTTTAGGACCGC 3'	Creation of the mutant P1M in HRV14VP12A (fwd)
TIM 1404	5' GGCCTAAACCCATGGATTTAAT GTCACCTTTCCTCTTCTTAATTA 3'	Creation of the mutant P1M in HRV14VP12A (rev)
TIM 1435	5' GGACATCAATCCGGAGCCGTGTATGTG 3'	Introduction of a <i>BspEI</i> restriction site into CVB4 VP1 2A ^{pro} (fwd)
TIM 1436	5' CACATACACGGCTCCGGATTGATGTCC 3'	Introduction of a <i>BspEI</i> restriction site into CVB4 VP1 2A ^{pro} (rev)
TIM 1437	5' GATGTTGAGGCGGTTACCGCGGAGCGTGCA 3'	Introduction of a <i>BstEII</i> restriction site into CVB4 VP1 2A ^{pro} (fwd)
TIM 1438	5' TGCACGCTCCGCGGTAACCGCCTCAACATC 3'	Introduction of a <i>BstEII</i> restriction site into CVB4 VP1 2A ^{pro} (rev)
TIM 1440	5' GATCTTAGCACCCGTGGGC CCCCAAGGCAAAACAAAGCGGTGTA 3'	Cassette for the creation of the mutant LSTR GPPR in PV1 (fwd)
TIM 1441	5' TACACCGCTTTGTTTTGC CTTGGGGGCCACGGGTGCTAA 3'	Cassette for the creation of the mutant LSTR GPPR in PV1 (rev)
TIM 1442	5' GTTACCGCGGAGCGGACAACCCTTAG CACCCGTGGGCCCCCAAGGCATCAAT 3'	Cassette for the creation of the mutant RTTLSTR GPPR in CVB4 (fwd)
TIM 1443	5' CCGGATTGATGCCTTGGGGGCCAC GGGTGCTAAGGTTGTCCGCTCCGCG 3'	Cassette for the creation of the mutant RTTLSTR GPPR in CVB4 (rev)
TIM 1479	5' AATTGTGACCAGACCACTTTGTTGAATGTTGGG TCACGAAGAATGTATGTTTCATGTAG 3'	Cassette for the creation of the mutant PLLNV GSRR in HRV2 (fwd)
TIM 1480	5' GTTACCTACATGAACATACATTCTTCG TGACCCACCATTCAACAAAGGTGGTCTGGTCAC 3'	Cassette for the creation of the mutant PLLNV GSRR in HRV2 (rev)
TIM 1481	5' CCGGTAATTAAGAAGAGGAAA GGTCCTTTGTTGAATGTTGGGTCACGACCGC 3'	Cassette for the creation of the mutant PLLNV GSR in HRV14 (fwd)

TIM 1482	5' GGTCGTGACCCAACATT CAACAAAGGACCTTTCCTCTTCTTAATTA 3'	Cassette for the creation of the mutant PLLNV GSR in HRV14 (rev)
TIM 1483	5' AATTGTGACCAGACCAGACATTAAA TCCTATGGTTTAGGACCGATGTATGTTTCATGTAG 3'	Cassette for the creation of the mutant DIKSY GLGP in HRV2 (fwd)
TIM 1484	5' GTTACCTACATGAACATACATCGGTCCT AAACCATAGGATTTAATGTCTGGTCTGGTCAC 3'	Cassette for the creation of the mutant DIKSY GLGP in HRV2 (rev)
TIM1485	5' CCGGTAATTAAGAAGAGGAAGGTATTATCAC TACAGCTGGCCCCAGTCCGC 3'	Cassette for the creation of the mutant IITTA GPSP in HRV14 (fwd)
TIM1486	5' GGACTGGGGCCAGCTGTAGTGATA ATACCTTTCCTCTTCTTAATTA 3'	Cassette for the creation of the mutant IITTA GPSP in HRV14 (rev)
TIM 1524	5' CCGGTAATTAAGAAGAGGAAAGGTCCTTTGTT GAATTATGGGTCACGACCGC 3'	Cassette for the creation of the mutant PLLNY GSR in HRV14 (fwd)
TIM 1525	5' GTTCGTGACCCATAATTCAACAAAGGACCTTT CCTCTTCTTAATTA 3'	Cassette for the creation of the mutant PLLNY GSR in HRV14 (rev)
TIM 1534	5' CCGGTAATTAAGAAGAGGAAAGGTC CTTTGTTGAATTATGGCTCGAGACCGC 3'	Cassette for the creation of the mutant PLLNY GSR in HRV14 with <i>AvaI</i> restriction site (fwd)
TIM 1535	5' GGTCTCGAGCCATAATTCAACAAAGGA CCTTTCCTCTTCTTAATTA 3'	Cassette for the creation of the mutant PLLNY GSR in HRV14 with <i>AvaI</i> restriction site (rev)
TIM 1536	5' CCGGTAATTAAGAAGAGGAAAGGTATTAT CACTACAGCTGGCCGAGTCCGC 3'	Cassette for the creation of the mutant IITTA GPSP in HRV14 with <i>AvaI</i> restriction site (fwd)
TIM 1537	5' GGACTCGGGCCAGCTGTAGTGATAATACCTTT CCTCTTCTTAATTA 3'	Cassette for the creation of the mutant IITTA GPSP in HRV14 with <i>AvaI</i> restriction site (rev)
TIM 1538	5' AATTGTGACCAGACCACCTTTGTTGAATGTT GGCTCGAGAAGAATGTATGTTTCATGTAG 3'	Cassette for the creation of the mutant PLLNV GSRR in HRV2 with <i>AvaI</i> restriction site (fwd)
TIM 1539	5' GTTACCTACATGAACATACATTCTTCTCGAG CCAACATTCAACAAAGGTGGTCTGGTCAC 3'	Cassette for the creation of the mutant PLLNV GSRR in HRV2 with <i>AvaI</i> restriction site (rev)
TIM 1540	5' AATTGTGACCAGACCAATTATCACTACAATGGG CCCAGTGACATGTATGTTTCATGTAG 3'	Creation of the mutant A M with <i>AvaI</i> restriction site (fwd)
TIM 1541	5' GTTACCTACATGAACATACATGTCACTC GGGCCCATGTAGTGATAATTGGTCTGGTCAC 3'	Creation of the mutant A M with <i>AvaI</i> restriction site (rev)
TIM 1542	5' AATTGTGACCAGACCAGACATTAAATCA TATGGTTTAGGACCGATGTATGTTTCATGTAG 3'	Cassette for the creation of the mutant DIKSY GLGP in HRV2 with <i>NdeI</i> restriction site (fwd)
TIM 1543	5' GTTACCTACATGAACATACATCGGTCCTAAA CCATATGATTTAATGTCTGGTCTGGTCAC 3'	Cassette for the creation of the mutant DIKSY GLGP in HRV2 with <i>NdeI</i> restriction site (rev)
TIM 1312	5' ATACATCAAGGTTTAGTG 3'	Sequencing of HRV14 VP1 2A ^{pro} (fwd)
TIM 1332	5' CATGCCATGGCCTGTTCTCTGCGATACA 3'	Sequencing of HRV14 VP1 2A ^{pro} (rev)
TIM 1333	5' CATGCCATGGGCCCCAGTGACATGTATGT 3'	Sequencing of HRV2 VP1 2A ^{pro} (fwd)

TIM 1323	5' TTTGGGATAGTACGGACTTTCCTGTAT 3'	Sequencing of HRV2 VP1 2A ^{pro} (rev)
TIM 550	5' GGACGTGGTTTTTCCTTTG 3'	Sequencing of pCITE- 1a IRES (fwd)
TIM 554	5' ATTTAGGTGACACTATAG 3'	Sequencing of pCITE- 1a SP6 (rev)
TIM 1196	5' GGATGTCCATACCGTTCATG 3'	Sequencing of CVB4 VP1 2A ^{pro} (fwd)
TIM 1098	5' ACTCACTAGAAGATCTCTATTATAATC 3'	Sequencing of CVB4 VP1 2A ^{pro} (rev)
TIM 1350	5' GACCAAGGTCACCTCCAA 3'	Sequencing of PV1 VP1 2A ^{pro} (fwd)
TIM 1351	5' TCCATGGCTTCTTCTTCG 3'	Sequencing of PV1 VP1 2A ^{pro} (rev)
TIM 592	5' ACAGACCATGGTTGAGAATTATATAGATG 3'	Sequencing of HRV2 VP1 2A ^{pro} (fwd)
TIM 1505	5' TGTAATTGGGAAGTATCTATTTTTATG 3'	Sequencing of HRV2 VP1 2A ^{pro} (rev)
TIM 1506	5' GTTAGAGTAGTCAATGATCACAACCCG 3'	Sequencing of PV1 VP1 2A ^{pro} (fwd)
TIM 1507	5' TTCGTAGGCATACAAGTCTCTAAT 3'	Sequencing of PV1 VP1 2A ^{pro} (rev)

Tab.6. Nucleotide sequences of all used primers and their function

2.2. Methods

2.2.1. Preparation of competent cells

Competent Top10F' cells were prepared using the CaCl_2 method. To this end, 200 μl of bacteria were inoculated in 3ml LB at 37°C over night. Following this, the culture was grown in 200ml of LB to an OD_{600} of 0.6. The bacteria were harvested in sterile tubes at 5000rpm for 5min. at 4°C. The medium was discarded and the cells were resuspended in 25ml of ice-cold 0,1M CaCl_2 and left in ice for 25min. Then, they were centrifuged at 5000rpm for 5 min. at 4°C, the medium was discarded again and the cells were resuspended in 10ml of 0.1M ice-cold CaCl_2 and left at 4°C over night. Finally, 2ml of sterile 80% glycerol were added and aliquots of 200 μl were frozen in liquid nitrogen. The competent cells were stored at -80°C.

2.2.2. Transformation of competent cells

A 200 μl aliquot of competent cells was thawed on ice for 10 min, then the DNA was added and left on ice for 15min. 10 μl DNA from a ligation mix or 1 μl DNA from a midi prep were used. Following this, the cells were heat shocked at 42°C for 45 seconds and transferred to ice immediately. 400ml of prewarmed LB were added and the mix was incubated at 37°C for 30 minutes. Following this, the samples were plated on agarose plates containing ampicillin. The plates were incubated at 37°C over night.

2.2.3. DNA preparation

Mini preps

Mini preps are used for the quick isolation of a larger amount of plasmid DNA of transformed bacteria from over night cultures that were grown in 3ml of LB_{AMP} . This method allows checking for positive clones after transformation. Positive clones can be identified by digestion with restriction enzymes or DNA sequencing.

3 ml of bacteria culture were grown over night in LB_{AMP} (100 $\mu\text{g/ml}$) at 37°C. 1,5ml of the culture were transferred to eppendorf tubes and spinned down at 13000rpm for 30 sec. The supernatant was discarded and the previous steps were repeated with another 1.5ml of

bacterial culture. Following this, the pellet was resuspended in 100µl of buffer S1, then treated with 200µl of lysis buffer S2, mixed gently and incubated at room temperature for 3 min. 150µl of neutralizing buffer S3 were added and the sample was mixed gently but thoroughly again. The sample was spun down at 13000rpm for 5 min. at 4°C and the supernatant was transferred to a clean eppendorf tube. 1ml of ice-cold ethanol was added and spinning at 4°C was repeated. The pellet was resuspended in 200µl of water and 200µl of 5M LiCl were added and centrifuged again. The supernatant was transferred to a new tube, 1ml of ice-cold EtOH was added and the sample was spun down at 4°C again. Following this, the pellet was air dried and resuspended in 50µl of water.

All used buffers were taken from the Nucleobond AX kit for Plasmid DNA preparation.

Midi preps

Midi preps are used to isolate larger amounts of very pure plasmid DNA that can be used for further cloning experiments or *in vitro* transcription.

100 ml of bacteria culture were grown over night at 37°C in LB_{AMP} (100µg/ml) and then the plasmid DNA was isolated using the Nucleobond AX kit for Plasmid DNA preparation.

The bacteria were harvested at 5000rpm for 5min. at 4°C, the supernatant was discarded and the pellet was resuspended in 4ml of buffer S1. 4ml of buffer S2 were added and the sample was incubated for 3 min. Following this, 4 ml of buffer S3 were added, the sample was mixed and centrifuged at 10000rpm for 25min. at 4°C. The lysate was loaded onto a column that had been equilibrated with buffer N2. The column was washed with 10ml of washing buffer N3 and then the sample was eluted with 5ml of buffer N5. 3,5ml of isopropanol were added and the sample was centrifuged at 11000rpm for 30min. at 4°C. The supernatant was discarded, 2ml of 70% were added to the pellet and then it was centrifuged at 11000 rpm for 10min. The pellet was air dried and the DNA was reconstituted in 200µl of H₂O.

2.2.4. DNA purification

DNA or RNA that has been contaminated or treated with enzymes has to be purified. During purification, proteins and salts can be removed. Two methods of DNA purification were used.

Phenol- chloroform- extraction

2µl of EDTA were added to the sample to stop enzyme reactions. Then 1 volume of phenol was added, the sample was vortexed and centrifuged at 13000rpm for 2min. The supernatant was transferred to a new tube, 1 volume of Chloroform was added, the sample was vortexed and centrifuged for 2 min. again. The extraction was followed by the Na- acetate precipitation. For that purpose 2 volumes of absolute EtOH and 10% of Na- acetate were added and the sample was incubated at -80°C for 20min. The sample was centrifuged at 13000rpm for 15min. at 4°C, the pellet was air dried and resuspended in 50µl water or TE buffer (10mM Tris, 1mM EDTA, adjusted to pH 7.5 using HCl).

DNA clean-up using Wizard SV gel and PCR clean-up system

1 volume of binding solution is added to the sample, the mixture is transferred to a column, incubated for 1 min. and centrifuged at 13000rpm for 1 min. The flow-through is discarded, 700µl of washing solution are applied onto the column and after 1min of centrifugation the flow-through is discarded again. 500µl of wash solution are added, and a centrifugation of 5min follows. The flow-through is discarded, the column is dried by 1min. centrifugation with the lid of the centrifuge opened. Finally the column is transferred into a new tube and the sample is eluted with 50µl H₂O.

2.2.5. Gel extraction of DNA fragments

Agarose gels (1%) are used to check the size and purity of DNA fragments resulting from analytical digests with restriction enzymes. In preparative digestions one of several fragments can be isolated from an agarose gel.

Gel extraction was performed using QIAquick- kit:

First, the DNA fragment was excised from the agarose gel and transferred to an eppendorf tube. 3 volumes of QG buffer were added and the mix was incubated at 50°C for 10min. in order to dissolve the agarose. After dissolution of the agarose 1 volume of isopropanol was added and the mix was loaded onto a column. The column was centrifuged at 13000rpm for 1 min., the flow-through was discarded and 500µl of QG buffer were added. After 1min.

centrifugation 750µl PE buffer were added, the mix was centrifuged again and the column was transferred to a new tube. The DNA was eluted with 50µl of H₂O.

2.2.6. Enzyme reactions

Restriction digests

For all restriction digests enzymes and 10x buffers of New England Biolabs (NEB) were used.

The following set-ups were used:

Analytical digestion: 5µl DNA (Mini prep)
2µl NEB buffer (10x)
0.5µl enzyme [20 000 U/ml]
12.5µl H₂O

20µl total volume

Preparative digestion: 30µl DNA (Midi prep)
10µl NEB buffer (10x)
1.5 µl enzyme [20 000U/ml]
58.5 µl H₂O

100µl total volume

Dephosphorylation

Dephosphorylation of the template is performed prior to ligation in order to prevent religation of the template. This is especially important in blunt end ligation. This was done with CIP (*Calf intestinal alkaline phosphatase*).

0.5ml of 2M Tris- HCl (pH 8.0) were added to 48.5µl of the restricted and purified DNA. The mix was incubated with 1µl of CIP (10U/µl, ® NEB) at 37°C for 45min., then CIP was inactivated at 65°C for 45min.

Kinasing oligos

The oligos that are used for a ligation are treated with PNK T4 (*Polynucleotide kinase T4*). This is done by PCR (*Polymerase chain reaction*) of a mix containing:

2µl ATP (100mM)
2µl PNK buffer (10x, Fermentas)
1µl oligo 1 (1µg/µl, VBC Biotech)
1µl oligo 2 (1µg/µl, VBC Biotech)
1µl PNK T4 (10000 U/ml, Fermentas)
13µl H₂O

The following PCR settings were used:

37°C 30min.
90°C 30sec.
37°C 5min.
4°C ∞
1 cycle

Ligation

The enzyme ligase is capable of connecting two pieces of DNA that have been restricted with the same enzymes. Therefore it is crucial in cloning an insert into a vector.

For all experiments T4 DNA ligase (10U/µl, ® NEB) was used. For a 20µl ligation mix of vector and insert 0.5µl T4 DNA ligase, 2µl T4 ligase buffer (10x) and 2µl of 100mM ATP were added. The ligation was incubated over night at room temperature, and then the sample was stored at 4°C until it was used for transformation.

2.2.7. Cassette cloning

Cassette cloning is a method that allows the rapid insertion of mutations into a vector. Two oligos of opposite sense that carry the mutation aimed on are connected and kinased (see 2.2.6). The oligo cassette is then ligated with the readily cut vector.

2.2.8. Site directed mutagenesis

For investigation of protein structure and function, the substitution of one or more amino acids is an important feature. While substitutions of several nucleotides are performed with cassette cloning, the substitution of up to three nucleotides can be performed with site directed mutagenesis.

This method uses a cycle PCR that amplifies the DNA sample. The oligos are designed in a way so the amino acid that shall be exchanged is located in the middle of them. As said before a mismatch of up to 3 nucleotides can be tolerated. Therefore, the oligos should contain approximately 10 nucleotides on each side of the mismatch. A negative control without the oligos has to be performed as well.

Following the PCR, the sample was digested with *DpnI* at 37°C for at least 5 hours. *DpnI* is a restriction enzyme that cleaves a sequence of 4 nucleotides, but only recognized this sequence when it is methylated. This means that it only digests the template strain, whereas the newly synthesized strain remains unaffected. After this digestion, the PCR product is transformed into Top10F' competent bacteria.

The following set up has been used:

- 1µl template DNA
- 0.5µl primer 1 (fw) (1µg/µl)
- 0.5µl primer 2 (rv) (1µg/µl)
- 2µl dNTPs
- 5µl PFU buffer (10x, Promega)
- 0.5µl PFU polymerase (Promega)
- 40.5µl H₂O

PCR- settings:

- 95°C 2min.
- 95°C 30sec.
- 50°C 1min.
- 68°C 12min.
- 4°C ∞
- 35 cycles

Where no PCR product was found, the annealing temperature was lowered to 50°C. The duration of the annealing was calculated from the length of the plasmid, i.e. 2min. per kb + 2 additional minutes. For CVB4 with a length of about 5kb that results in 12min. annealing.

Site directed mutagenesis and cassette cloning are compared in Fig.9.

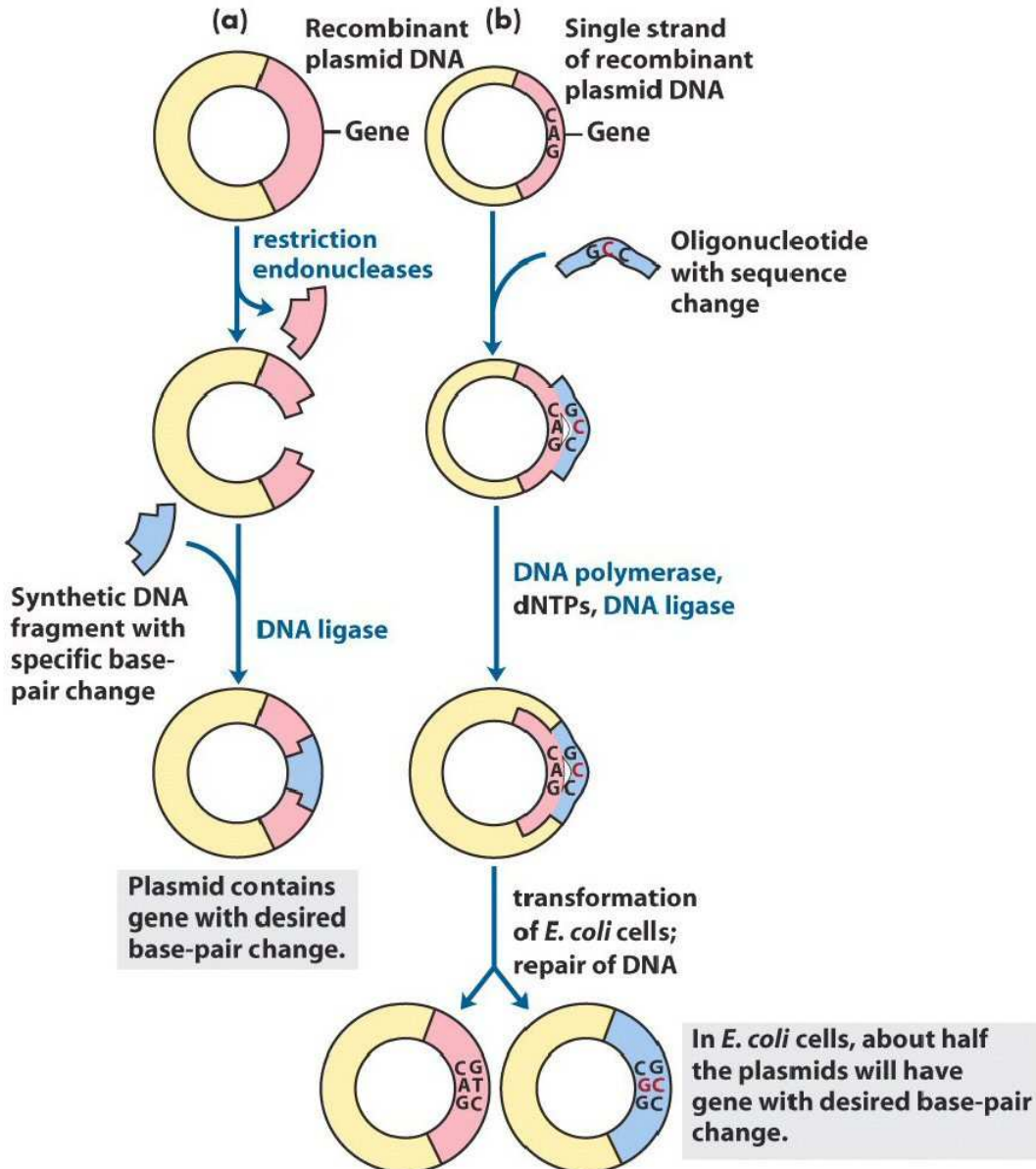


Fig. 9: Comparison of cassette cloning and site directed mutagenesis

(a) shows the steps of cassette cloning: First, the vector is cut with two restriction enzymes in order to remove the part of the DNA in which the mutation will be inserted. Two oligos of opposite sense that carry the mutation aimed on are connected and kinased. The DNA fragment carrying the mutation and the vector are then joined by ligase. (b) shows the introduction of a mutation by site directed mutagenesis. In this method a cycle PCR is used to amplify the DNA sample. Two oligos are designed in a way so the amino acid that shall be exchanged is located in the middle of them. Low annealing temperatures allow that the mutation is introduced into the newly synthesized strands. Following this, the PCR mix is digested with DpnI in order to remove the template DNA. Figure 9 has been extracted from [6].

2.2.9. *In vitro* transcription

For the production of RNA, the template must be first linearized. pCITE-1a VP1 2A^{pro} of HRV2 and HRV14 were linearized with the enzyme *Bam*HI, whereas *Pst*I was used for the linearization of CVB4 and *Sac*I for PV1. For all linearizations, about 5µg template DNA were used. 20 to 40µl template were mixed with 10µl NEB buffer (10x) and 1µl enzyme and replenished with H₂O to 100µl. The mix was incubated for at least 3 hours at 37°C. When linearization was incomplete after 3 hours, longer incubation times were used. After linearization the product was purified.

Transcription mix (100µl total):

50 µl purified template DNA
20µl transcription buffer (5x)
5µl DTT (100mM)
10µl NTP mix (2,5mM)
3µl RNAsin
1µl T7 polymerase
11µl H₂O (RNase free)

The mix was incubated for 90 min. at 37°C, then 0.6µl DNaseI (30 units) and 1µl RNAsin (40 units) were added and incubation at 37°C was continued for 20 more minutes. Following this, the RNA was purified using phenol/chloroform- extraction. Instead of sodium acetate precipitation an ammonia acetate precipitation was carried out. After extraction, 2.5 volumes of 96% EtOH and 1/3 volume of NH₄Ac (8M) were added and the mix was incubated at -80°C for 15min. The precipitated RNA was resuspended in 25µl RNase free water. The amount and quality of the RNA were checked on a 1% agarose, 0.1% SDS gel.

2.2.10. *In vitro* translation

For all *in vitro* translation experiments rabbit reticulocyte lysates (RRL) were used. In order to check whether the mutants are capable of self processing and to find the exact time point at which self processing takes place, time course experiments were run. For short range time courses the time points 0, 5, 10, 20, 30 and 60 minutes were examined, for long range experiments the time points were set at 30, 60, 90, 120 and 180 minutes. The total volume of

the translation mix was 10 μ l for one time point. For each time point observed 1 volume of the translation mix was added. The appropriate RNA amount was checked previously by translating several dilutions of RNA.

Translation mix (10 μ l):

7 μ l RRL
0.2 μ l Amino acid mix – methionine
0.2 μ l RNAsin
0.4 μ l ³⁵S labelled- methionine
1.2 μ l H₂O
1 μ l RNA dilution

The translation mix was incubated for 2 min at 30°C without RNA, then the RNA was added. At each time point one volume of translation mix (10 μ l) was stopped by adding the mix to 41 μ l of ice cold stop mix, consisting of 1 μ l methionine/cysteine mix (20mM), 25 μ l Laemmli sample buffer (2x) and 15 μ l H₂O. The 5x Laemmli sample buffer consists of 50%glycerol, 25% β - mercapto- ethanol, 15% SDS, 315mM Tris HCl (pH 6.8) and 25% (w/w) bromophenol blue.

The translation product was separated by SDS- PAGE.

2.2.11. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS- PAGE is a widely used technique for separating proteins according to their size, which is their mass in kD. For this thesis the polyacrylamide gel electrophoresis system described by Dasso & Jackson (1989) was used for analysis of translation products. The samples are boiled for 5 minutes for denaturation prior to loading the gel. The β - mercapto- ethanol contained in the Laemmli sample buffer reduces disulfide bridges, so full denaturization is guaranteed. The SDS adds a strong negative charge to the protein so the length of the protein corresponds to the protein mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in folding, which would make some proteins fit better through the gel matrix than others. With SDS added the proteins are negatively charged. This allows separating the proteins only according to their mass. The grade of interlacing of the

acrylamide is also designated by the size of the polypeptide and its cleavage product. In this case 17.5% acrylamide gels were used.

Resolving gel:

1020 μ l acrylamide
209 μ l bisacrylamide
1050 μ l LGS buffer (4x)
750 μ l H₂O
30 μ l APS (10%)
3 μ l TEMED

A stacking gel was placed on top of the resolving gel. This stacking gel has larger pores and a different pH, so the proteins are able to migrate fast and without being separated through the stacking gel. When they reach the interface between stacking gel and resolving gel their migration is decelerated considerably and the new migration speed is now corresponding to the size of the proteins resulting in separation.

Stacking gel: (stock)

6.5 ml acrylamide
12.5 ml UGS buffer (4x)
31 ml H₂O

For each gel 2ml of the stock are used and 12 μ l APS (10%) and 4 μ l TEMED are added. LGS buffer consists of 1,5M Tris and 0,4% SDS, pH 6,8, UGS buffer has the same composition, but the pH adjusted to 8,8.

The readily prepared gel is then loaded with the samples. The 10x running buffer contains 250mM Tris, 2 M glycerol and 1% SDS.

The detection of the radioactively labelled translation product was done by fluorography (Laskey, 1980). Therefore the stacking gel was washed 2 times 15minutes in enhancing buffer after running. The Enhancer consists of 32g sodiumsalicylate, 90ml methanol and 110ml H₂O and washes away the radioactive background while enhancing the right signals. After washing, the gels were dried on 3mm Whatman paper for 1hour at 80°C. Then a Kodak Biomax MR film was placed on the gel and the film was exposed for approximately 24 hours

at -80°C. As radioactive marker ^{14}C - CFA 626 (® Amersham Pharmacia Biotech) was used, which gives bands at 220, 97.4, 66, 46, 30, 14 and 3kDa. 5µl of the marker and 10µl of the samples were loaded on the gel.

3. AIMS

All members of the picornavirus family are small, non-enveloped icosahedral viruses with a single stranded RNA genome of positive sense. Their genomic information is translated into a single polyprotein of about 200 kDa that is cleaved to mature proteins by the viral proteases 2A and 3C. 2A Protease (2A^{pro}) exerts the first cleavage in rhino- and enteroviruses and separates structural from non-structural proteins (Toyoda *et al.*, 1986). In order to do so, it cuts between its own N-Terminus and the C-Terminus of capsid protein VP1. Following this, 2A^{pro} cleaves between the two isoforms of eukaryotic initiation factor 4G (eIF4G), a translation factor of the host cell that plays an important role in cap- dependent translation. This second cleavage leads to a shut-off of host- cell translation, whereas viral translation is not affected as it is cap- independent.

Several picornaviruses are known to be the causative agents of human and animal diseases which makes them an important issue for research. As the 2A^{pro} is highly conserved among rhino- and enteroviruses it is a potential target for antiviral drugs. In order to develop effective inhibitors an understanding of the substrate specificity of the picornaviral proteases is highly important.

Proteins of four members of the Picornavirus family were examined in this thesis: human rhinovirus (HRV) 2 and 14 are two of more than 100 serotypes of human rhinoviruses, which are the main causative agent of common cold (Couch, 1990), a disease that is reported to be an important economical factor as it causes absences from work (Bramley *et al.*, 2002; Fendrick *et al.*, 2003). The 2A^{pro} of the two rhinoviruses were chosen as they differ significantly in their amino acid sequence and the receptor they use (Skern *et al.*, 1985; Colonno, 1986). While HRV2 is an A- group rhinovirus of the minor group that uses LDL receptors, HRV14 belongs to the B- group and is a major group rhinovirus as it uses ICAM as receptor. The HRV2 and HRV14 2A^{pro} share only 40% amino acid sequence identity and show remarkable differences in the two 2A^{pro} cleavage reactions. This is especially true for the cleavage of eIF4G. Additionally one member of coxsackie viruses, Coxsackie virus B4 (CVB4) and one of poliovirus, PV1, were examined. Coxsackie B viruses are reported to be related with internal diseases (Kayser *et al.*, 1998) and are a cause of myocarditis, which is connected with congestive cardiomyopathy, a severe disorder of the heart (Liu *et al.*, 1993). Furthermore, CVB4 is a possible cause of Diabetes mellitus type I. CVB4 is an enterovirus with about 40% sequence identity with HRV2. Structural identity is also given for large parts.

Poliovirus 1 (PV1), the causative agent of poliomyelitis, is also an enterovirus however, it shows some similarity to HRV14.

Although the sequences of the different picornaviral 2A proteases are known and structural predictions were made due to sequence alignments with known structures, a crystal structure is only available for HRV2 2A^{pro} (Petersen *et al.*, 1999). Recently, the solution structure of CVB4 2A^{pro} was published (Baxter *et al.*, 2006). The structures revealed some details about the mechanism and substrate specificities of the picornaviral 2A proteases, but still many questions about their function remain unanswered. In order to gain understanding of the activity of picornaviral self-processing and cleavage of eIF4G the cleavage specificities of HRV2, HRV14, PV1 and CVB4 2A^{pro} were examined and compared in this thesis.

The comparison of the activities of the 2A^{pro}s of these viruses was performed *in vitro* in rabbit reticulocyte lysate (RRL). The self-processing, that is the cleavage of the polyprotein, between 2A^{pro} and VP1 was assayed.

First, the amino acids at the P1 position in HRV2 and HRV14 were changed into a methionine in order to find out how this might affect the self-processing. This substitution was especially interesting as the same mutation enhanced the cleavage in bacteria. An increased cleavage efficiency has also been shown for synthetic peptides substituted with methionine at the P1 (Sommergruber *et al.*, 1992).

Following this, the cleavage site of HRV2 and HRV14 was replaced by a cassette with the sequence of eIF4GII. This was done to find out whether the cleavage site of eIF4GII could still be recognised in a changed environment. The data then could be compared to the results of the same experiments with insertion of eIF4GI. (Sousa *et al.*, 2006; Schmid, 2002).

The influences of a substitution of the cleavage site by the sequence of eIF4GI on the self-processing was then analysed for PV1 and CVB4. This was especially interesting as it could be compared with the results for HRV2 and HRV14. For these proteinases it had been shown that HRV2 2A^{pro} was able recognise and cleave eIF4GI when it was introduced between VP1 and 2A on the polyprotein, while HRV14 2A^{pro} was not (Sousa *et al.*, 2006). As the 2A^{pro} of HRV2 resembles that of CVB4 in some aspects and PV1 shows some similarity to HRV14, it was of great interest to examine how they were influenced by the introduction of eIF4GI into the polyprotein.

Additionally, the cleavage sites of HRV2 and HRV14 were interchanged in order to examine the effect on the self-processing. Given the low amino acid sequence identity of only 40% between the 2A proteases of HRV2 and HRV14 and the difference in the cleavage reactions it was an intriguing question whether the 2A^{pro} proteases would be able to recognise the significantly different cleavage site of the respective other 2A^{pro} when inserted between VP1 and 2A^{pro}.

4. RESULTS

4.1. Introduction of methionine at P1 position in HRV2 VP1 2A^{pro} and HRV14 VP1 2A^{pro}:

2A^{pro} executes the first cleavage reaction in picornaviruses, which is the cleavage between its own N-Terminus and the C-Terminus of VP1. It has been shown previously that substitution of the amino acid tyrosine at P1 position by methionine results in accelerated cleavage of the polyprotein. This was demonstrated in bacteria and with synthetic peptides. Now, it was to be tested whether this enhanced self-processing was also observed in a more sensitive system, that of the rabbit reticulocyte lysate.

To examine the efficacy of self processing from the polyprotein, the mutants HRV2 VP1 2A^{pro} P1M and HRV14 VP1 2A^{pro} P1M were constructed. Then, the plasmid DNA was prepared, linearised and used for *in vitro* transcription. A concentration of approximately 10ng/μl RNA was used. A time course of translation was carried out using 0min, 5min, 10min, 20min, 30min and 60min as time points. The wild type constructs HRV2 VP1 2A^{pro} wt and HRV14 VP1 2A^{pro} wt were used as positive controls, water as a negative control. After translation the proteins were boiled 2 min. at 95°C and 10μl aliquots loaded on a 17.5% polyacrylamide gel and analysed by fluorography.

In wild type HRV2 VP1 2A^{pro} self-processing the first protein bands are visible after 10min. After 20min, 50% of the polyprotein is cleaved and after 60min cleavage is complete (Fig.10). The uncleaved species has a size of approx. 49kDa; the band can be seen a little above the 46kDa marker. VP1 has a size of about 33.5kDa, the band is placed significantly above the 30kDa marker, which suggests a retarded migration. 2A^{pro} has a size of 15,5 kDa, the band can be seen above the 14kDa marker. The 2A^{pro} band is quite weak as it is difficult to detect by autoradiography as 2A^{pro} contains only two methionines, whereas VP1 contains eight.

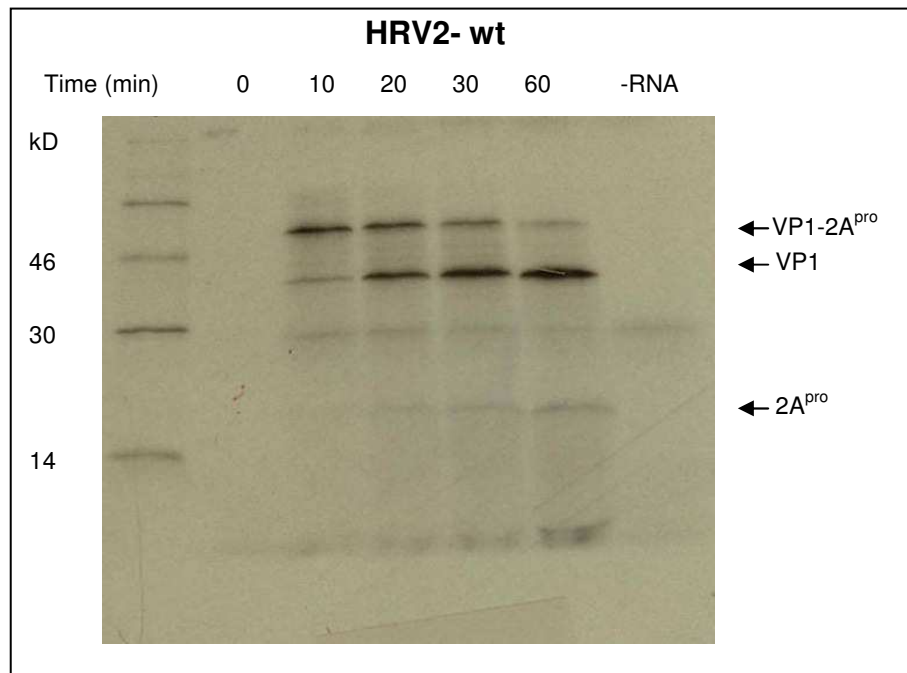


Fig.10:Self- processing of HRV2-VP1-2A^{pro} wild type during *in vitro* translation. RRL were incubated with the mRNA as described in “materials and methods”. Aliquots were taken at the set time points and translation stopped by addition of a 2mM mix of unlabelled methionine and cysteine and Laemmli buffer followed by placing the samples on ice. The aliquots were analysed by 17,5% SDS- PAGE to investigate the cleavage of VP1-2A^{pro}. The fluorogram was exposed at -80°C for 20 hours. The position of uncleaved VP1-2A^{pro} and the fragments of VP1 and 2A^{pro} after cleavage are marked. Radioactively labelled CFA- 626 (Amersham TM) was used as marker.

In the mutant HRV2 VP1 2A^{pro} A → M, the self-processing cleaves at wild type rates. The first protein bands are visible after 10min, 50% are cleaved after 20min and after 60min cleavage is completed (Fig.11). Whether the cleavage works better compared to the wild type, as observed in bacteria and with peptides, could not be observed in this translation experiment. Specific data on the kinetics of the cleavage would be necessary to investigate this.

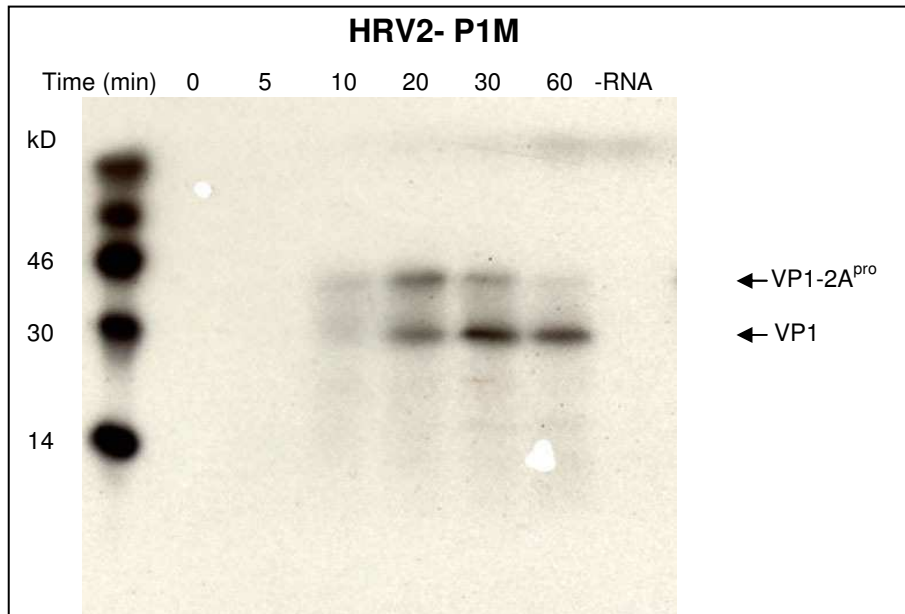


Fig.11: Self-processing during *in vitro* translation of HRV2-VP1- 2A^{pro} with the alanine at P1 changed into methionine. The mRNA was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} and the cleavage product VP1 are marked. After 20 min. 50% of VP1- 2A^{pro} are cleaved, after 60 minutes cleavage is completed. This efficiency corresponds to the cleavage of the wild type.

Figure 12 shows that the self processing performance in wild type HRV14 VP1 2A^{pro} is very similar to that in HRV2. Cleavage is only slightly delayed in comparison to HRV2, starts after 20min, is 50% complete after 30min and almost complete after 60min. 2A^{pro} is easier to detect in HRV14 as it contains three methionines, VP1 has six.

In the mutant HRV14 VP1 2A^{pro} Y → M the cleavage is slightly retarded compared to the wild type. Although the protease started to cleave itself out of the polyprotein after 20min like in the wt, less than 50% were cleaved after 30min and cleavage was not totally completed after 60min. This is shown in Fig.13.

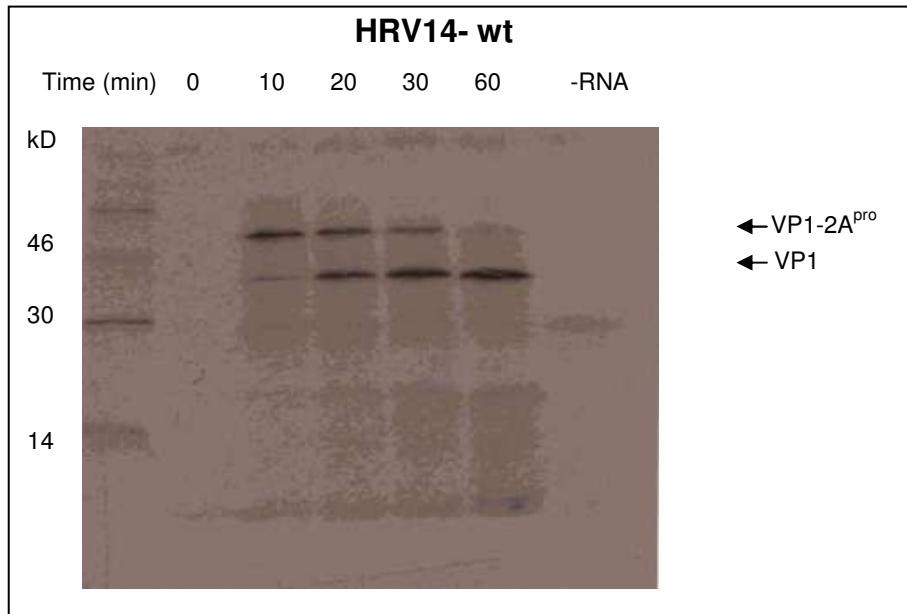


Fig.12: Self- processing of HRV14-VP1-2A^{pro} wild type during *in vitro* translation. The mRNA HRV14-VP1-2A^{pro} was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} and the cleavage products VP1 and 2A^{pro} are marked.

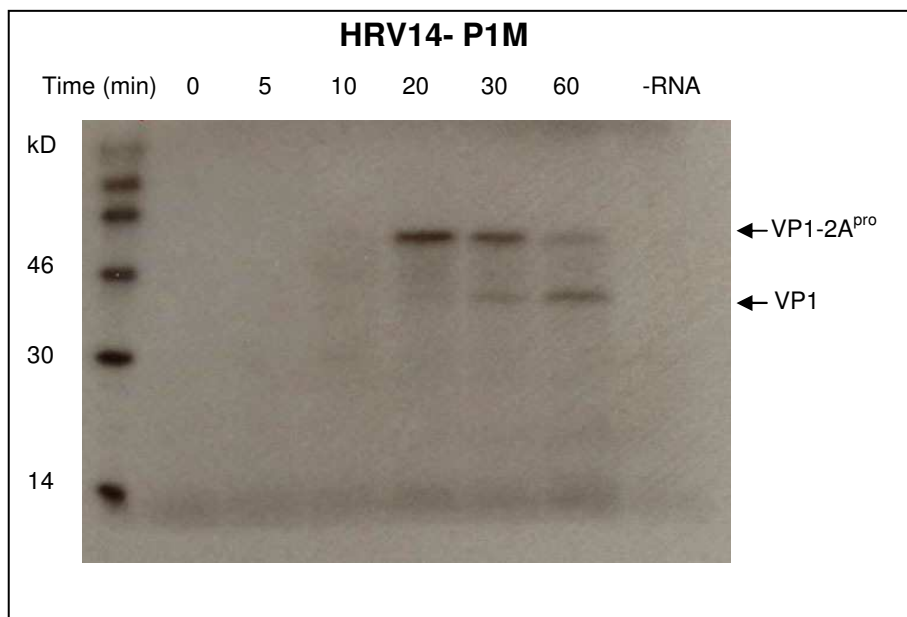


Fig.13: Self- processing during *in vitro* translation of HRV14-VP1- 2A^{pro} with the tyrosine at P1 changed into methionine. The self- processing of the mRNA HRV14-P1M was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} and the cleavage product VP1 are marked. Cleavage started after 20min, was less than 50% completed after 30 min and almost completed after 60min.

4.2. Insertion of the 2A^{pro} cleavage site of HRV14 into HRV2, and the 2A^{pro} cleavage site of HRV2 into HRV14:

HRV2 and HRV14 are two serotypes of human rhinovirus that differ significantly. HRV2 is a minor- group member, which means that it uses LDL- receptors, whereas HRV14 belongs to the major group viruses and use ICAM receptors. HRV2 and HRV14 show only 51% identity in their amino acid sequence, their 2A proteases are only 40% identical (Wang *et al.*, 1998). To find out what happens when the cleavage sites of HRV2 and HRV14 between VP1 and 2A^{pro} are removed from their original protein context, new mutants were created using cassette cloning.

Eight amino acids of the cleavage site of HRV2 VP1 2A^{pro}, IITTA * GPS; were replaced by the corresponding region of HRV14 VP1 2A^{pro}, DIKSY * GLGP and *vice versa*. After cloning of the mutants, the linearized templates were transcribed *in vitro*. Following this, the mRNAs were translated *in vitro* and translation was monitored by autoradiography to investigate whether the 2A^{pro} was still able to recognise the cleavage site and cut itself out of the polyprotein in spite of the mutated amino acid sequence.

Fig.14 shows that if the sequence of HRV14 is transferred to HRV2, no cleavage can be observed during 180 minutes of *in vitro* translation

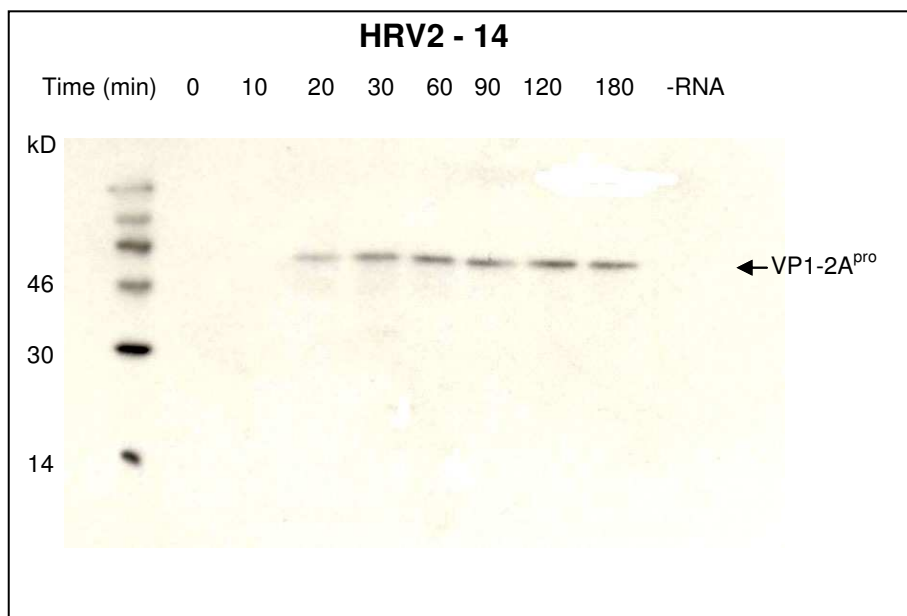
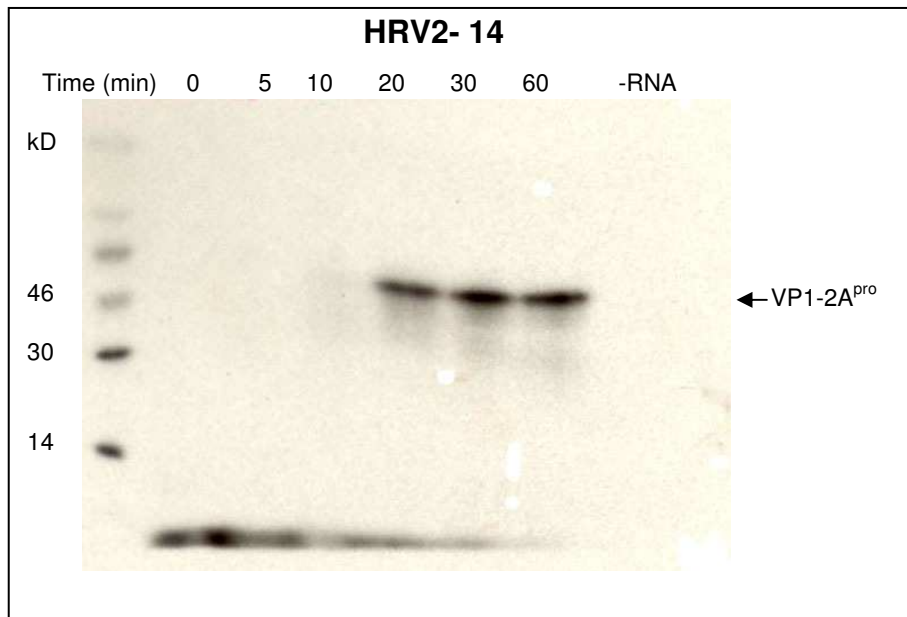


Fig.14: Self- processing of HRV2- VP1- 2A^{pro} with the cleavage site of HRV14-VP1-2A^{pro} inserted during *in vitro* translation. The cleavage of the polyprotein by 2A^{pro} was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} is marked. The polyprotein was not cleaved within 180 min.

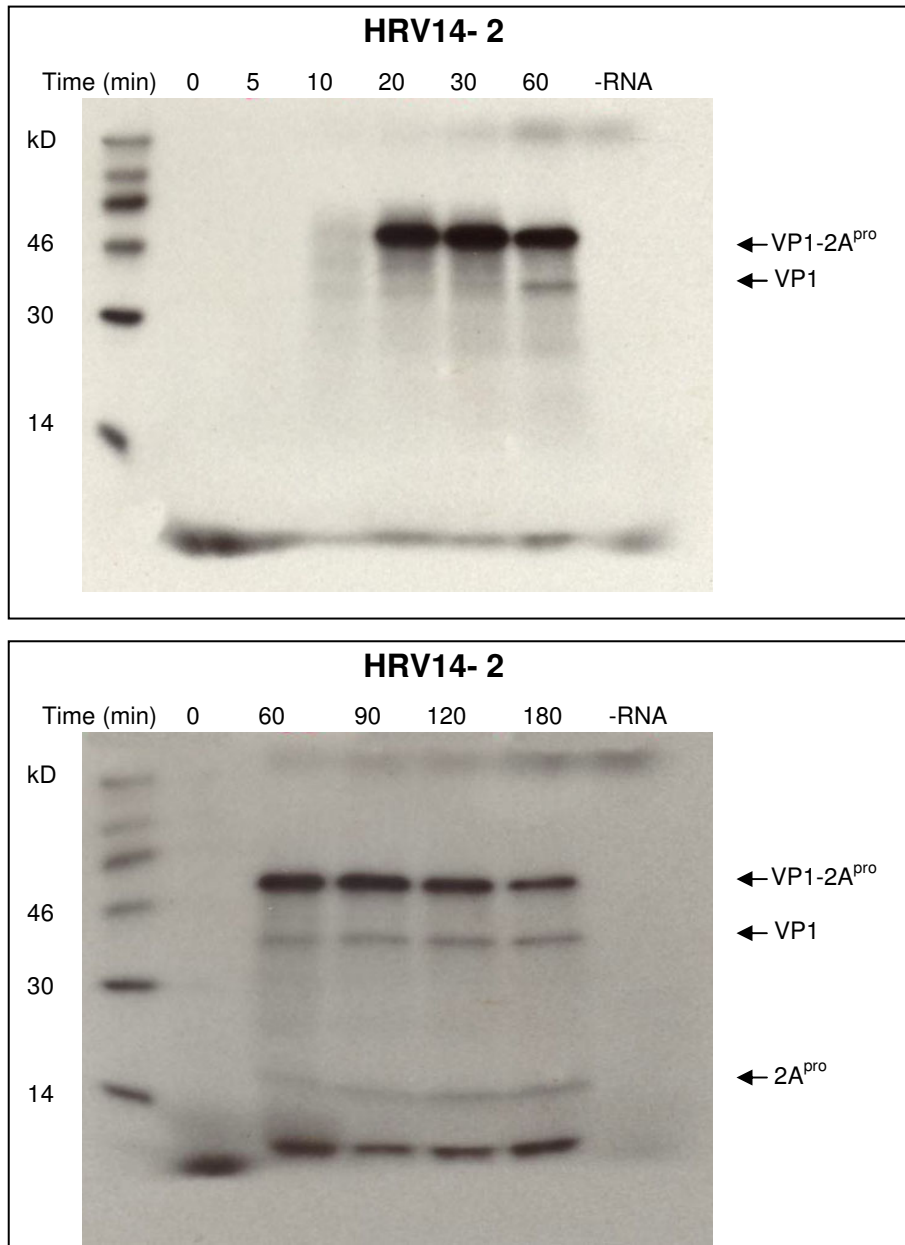


Fig.15: Self- processing of HRV14- VP1- 2A^{pro} with the cleavage site of HRV2- VP1- 2A^{pro} inserted during *in vitro* translation. The cleavage of the polyprotein by 2A^{pro} was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} and the cleavage product VP1 are marked. No cleavage could be observed before the 60 minutes time point. After 180 min. almost 50% of the polyprotein were cleaved.

Different results occurred when the cleavage site of HRV2 was transferred to HRV14, as can be seen in Figure 15. During the first 60 minutes again no cleavage was observed, but after 60 minutes translation started and after 180 minutes approximately 50% of the polyprotein were cleaved.

4.3. Insertion of the cleavage site of eIF4GII into HRV2 and HRV14:

The 2A protease of picornaviruses executes two cleavage reactions that are crucial in the viral life cycle. First, it cleaves itself out of the polyprotein during self-processing, as described in chapter 1.3. Secondly, it cleaves the two isoforms of eukaryotic initiation factor 4G (eIF4G) resulting in the shut-off of host cell translation. While self-processing proceeds very similar in HRV2 and HRV14, there are remarkable differences *in vivo* in the cleavage of the two isoforms of eIF4G. eIF4GI is cleaved significantly slower by HRV14 than by HRV2. eIF4GII is cleaved by HRV2 simultaneously with eIF4GI, whereas it is cleaved remarkably later by HRV14 during *in vitro* translation (Gradi *et al.*, 2003; Foeger *et al.*, 2003). It has been shown that *in vitro* cleavage of eIF4GI by HRV2 2A^{pro} is extremely efficient having a molar ratio similar to that observed during infection *in vivo* (Glaser & Skern, 2000). Due to the degradation of the RRL after 6 hours it is not clear whether eIF4GII is cleaved at all by HRV14 *in vitro*.

In order to gain information on the eIF4GI cleavage site, the sequence of the cleavage site was removed from its original protein surroundings and introduced into the polyprotein between VP1 and 2A^{pro}. Therefore, the sequences IITTA * GPSD and DIKSY * GLGP that are cleaved by HRV2 and HRV14 2A^{pro} on the respective polyprotein were replaced by the TLSTR * GPPR sequence that is cleaved on eIF4GI by HRV2 2A^{pro}. It was shown that the HRV2 2A^{pro} still was able to recognise the cleavage site and cut itself out of the polyprotein, while HRV14 was not able to do so due to the presence of arginine at P1 (Sousa *et al.*, 2006). Now we wished to examine, whether the same was true for the second isoform, eIF4GII.

The sequence of the cleavage site of VP1 2A^{pro} was therefore substituted by the cleavage site of eIF4GII, being PLLNV * GSRR. This was done by cassette cloning as explained in material and methods (2.2.7). Nine amino acids of the VP1 2A^{pro} cleavage site on the polyprotein of HRV2 and eight of HRV14 were replaced by the amino acids of the cleavage site of eIF4GII. During *in vitro* translation the 2A^{pro} of HRV2 and HRV14 now had to be able to recognise the eIF4GII cleavage site in *cis* in order to free themselves from of the polyprotein. After cloning the mutants they were again transcribed into RNA and then translated *in vitro*. The results of *in vitro* translation are shown in Fig.14.

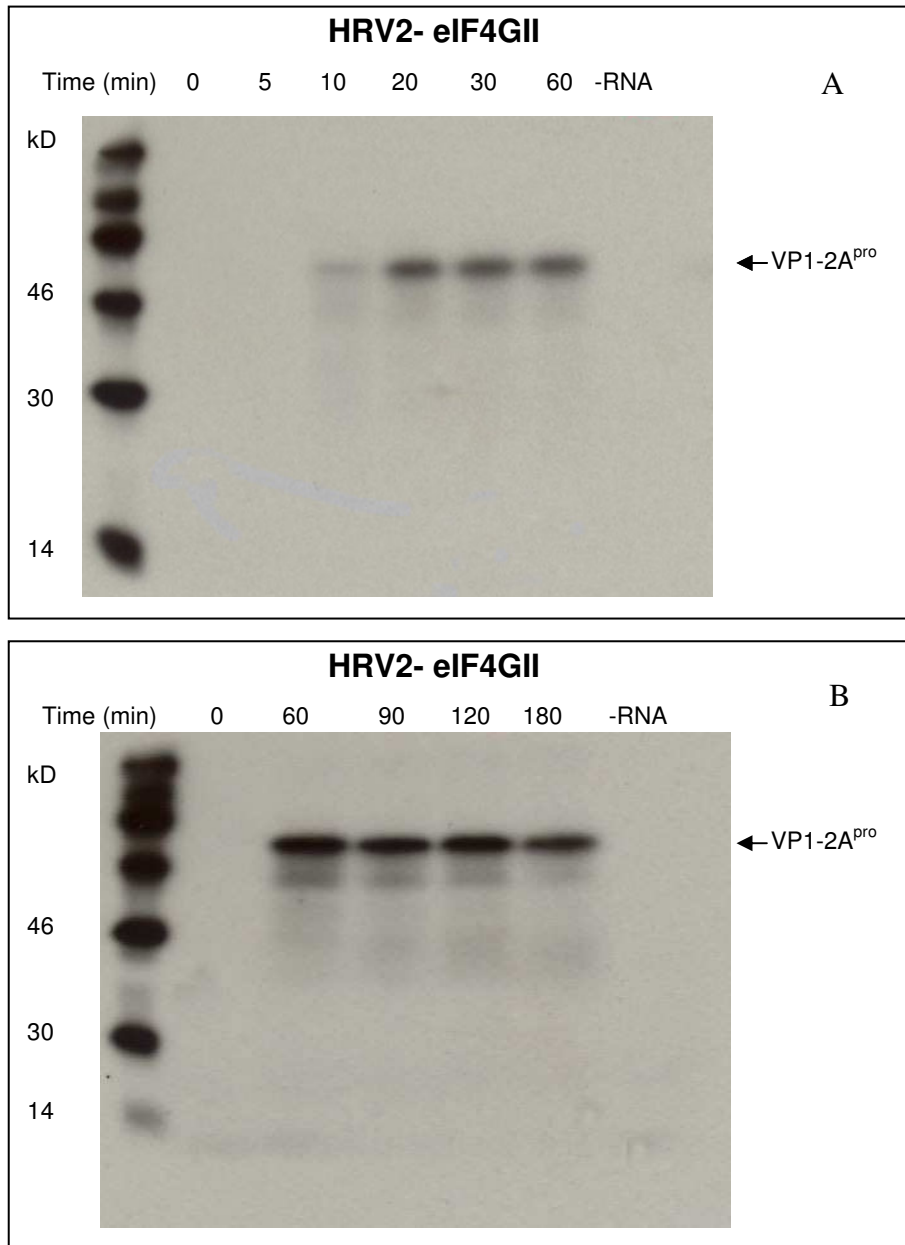


Fig.16: Self-processing of HRV2- VP1-2A^{pro} with insertion of the eIF4GII cleavage site during *in vitro* translation. Analysis was performed as described in Fig.10. Uncleaved VP1-2A^{pro} is marked. No self-processing could be examined after 60min (A). After 180min still only the uncleaved product is visible, no cleavage took place (B).

As can be seen in Figure 16a, no cleavage could be observed after 60min. Subsequently, a longer range time course was run, adding time points after 90min, 120min and 180min. Figure 16b shows that after 180min, cleavage had still not started, as only VP1-2A^{pro} was visible. The HRV2 2A^{pro} was therefore unable to recognise the eIF4GII cleavage site in a different protein context.

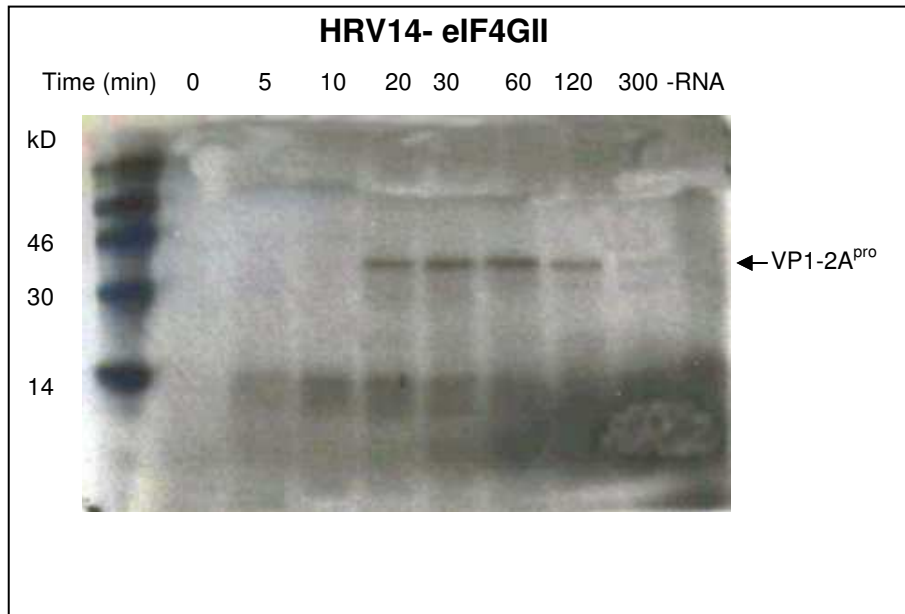


Fig.17: Self-processing of HRV14-VP1-2A^{pro} with the cleavage site of eIF4GII inserted during *in vitro* translation. The cleavage of the polyprotein was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} is marked. No cleavage occurred during 300 min of *in vitro* translation.

Figure 17 shows the results for HRV14. It can be seen that again no cleavage was executed after 60min. As for HRV2, a longer time course was run. No cleavage of eIF4GII could be examined, giving evidence that the 2A^{pro} of HRV14 is also not able to recognise the eIF4GII cleavage site in *cis*.

It could be shown that HRV14 was able to recognise eIF4GI in *cis*, when the arginine at the P1 position of eIF4GI was substituted by the tyrosine of the wild type cleavage site of HRV14 VP1 2A^{pro} (Sousa *et al.*, 2006). Thus we examined whether the same was true for the cleavage of eIF4GII. The valine at the P1 position of eIF4GII was substituted by tyrosine, as in the HRV14 wild type. The remainder of the eight amino acids of the eIF4GII cleavage site remained unchanged, creating the sequence PLLNY * GSRR. After transcription, the mutant RNA was *in vitro* translated in order to find out whether the substitution was sufficient to regain self-processing of the 2A^{pro}.

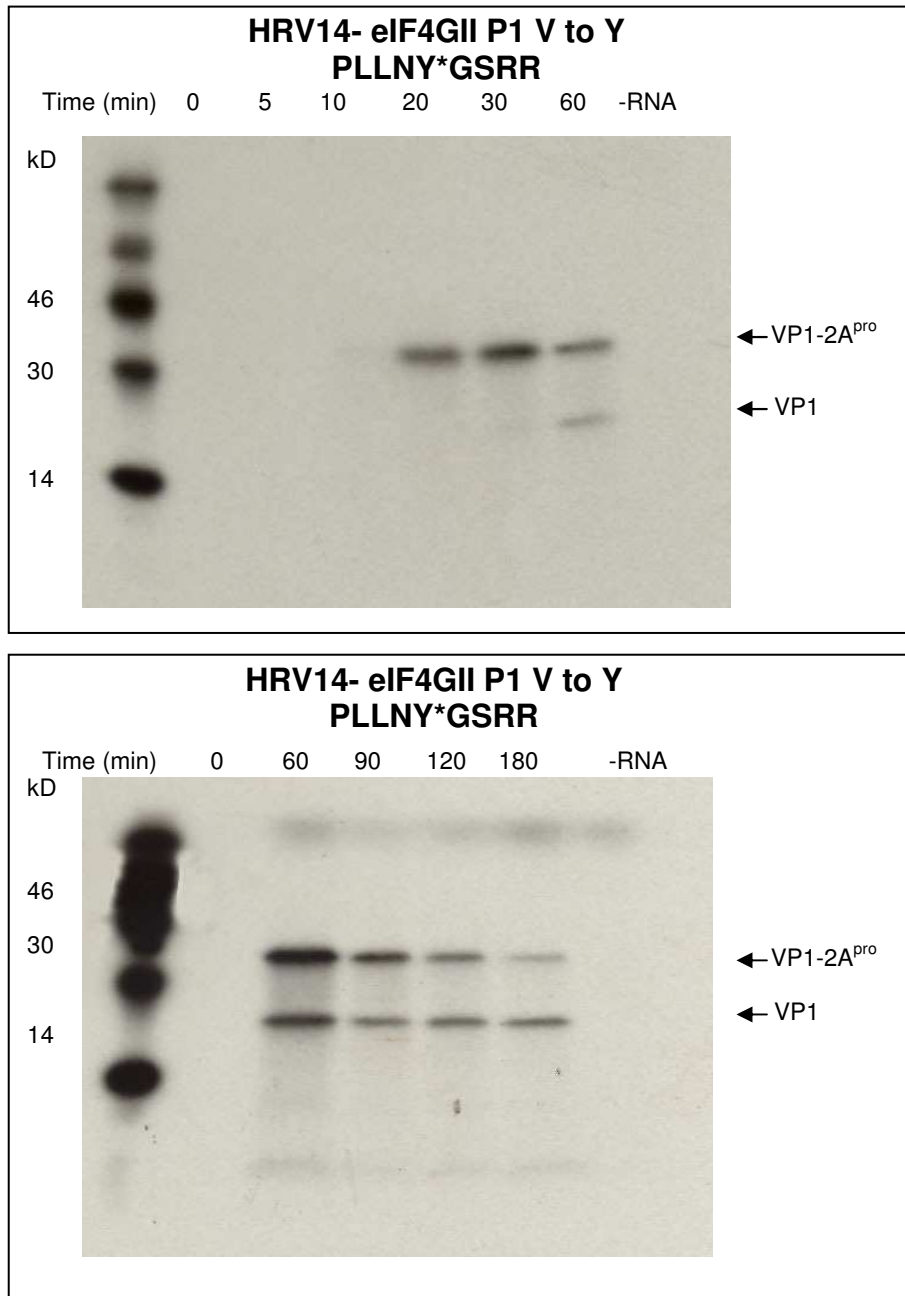


Fig.18: Self- processing of HRV14-VP1-2A^{pro} with the cassette PLLNY*GSR inserted during *in vitro* translation. The mRNA was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} and the cleavage product VP1 are marked. No cleavage could be examined for 60 min. At this time cleavage of the polyprotein started and was almost complete after 180 min.

Figure 18 shows that replacing the valine at P1 by tyrosine was indeed sufficient to regain the function of HRV14 2A^{pro} to a certain degree. *Cis* cleavage of the eIF4GII started after 60min and was almost completed after 180min.

4.4. Insertion of the cleavage site of eIF4GI into PV1 and CVB4:

As mentioned earlier, previous experiments examined how HRV2 and HRV14 2A^{pro} function when the cleavage site of eIF4GI is introduced instead of the original cleavage site. HRV2 was still able to recognise the cleavage site and cut eIF4GI in *cis*, whereas HRV14 could not. As the 2A^{pro} of all picornaviruses performs the cleavage of the two isoforms of eIF4G in *trans*, it was of interest whether other picornaviruses would be able to recognise the cleavage site in *cis*, as can be done by HRV2 2A^{pro}. Two picornaviruses, one serotype of poliovirus, PV1, and one of coxsackie virus, CVB4, were therefore examined.

The eight amino acids LTTY * GFGH of PV1 VP1 2A^{pro} and 11 amino acids RASLITT * GPYG of CVB4 VP1 2A^{pro} were replaced by the cleavage sequence of eIF4GI by cassette cloning, being LSTR * GPPR for PV1 and RTTLSTR * GPPR for CVB4 2A^{pro}. For CVB4 two restriction sites, *BstEII* and *BspEI*, had to be created to enable the introduction of the cassette. This was done by site directed mutagenesis. Again, the clones were *in vitro* transcribed into RNA and then translated *in vitro* in RRLs.

First, the differences in the *in vitro* translation of PV1 VP1 2A^{pro} wild type and CVB4 VP1 2A^{pro} wild type were examined. In Fig.19, it can be seen that the cleavage of PV1 VP1 2A^{pro} started after 20 minutes and was completed after 180 minutes. In case of CVB4 VP1 2A^{pro} 50% of the polyprotein were cleaved after 20 minutes, after 60 minutes cleavage was almost completed (Fig.20).

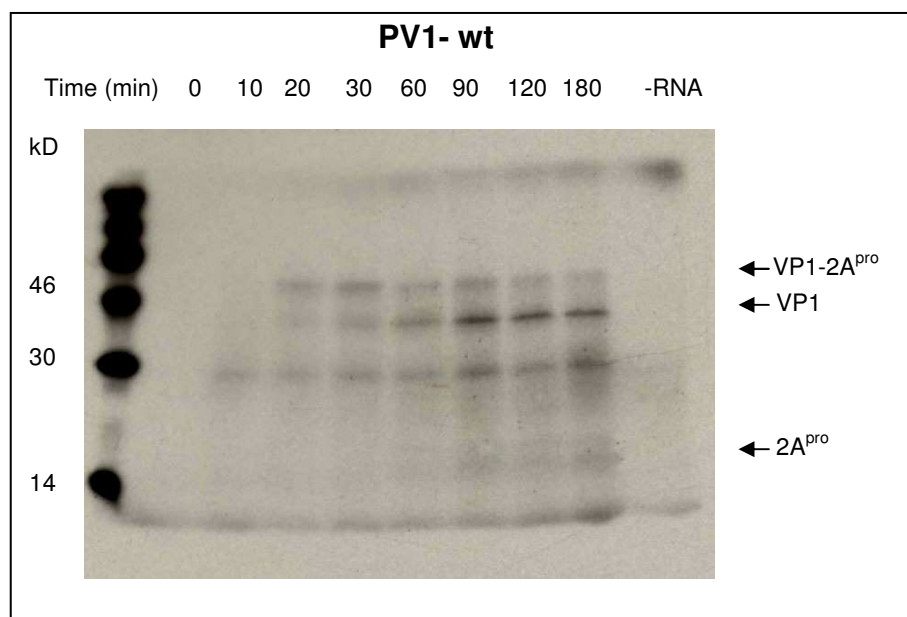


Fig.19: Self-processing of PV1- VP1- 2A^{pro} from the polyprotein during *in vitro* translation. The mRNA was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} and the cleavage products VP1 and 2A^{pro} are marked. Cleavage of the polyprotein started after 20 minutes translation but was not completed after 180 min.

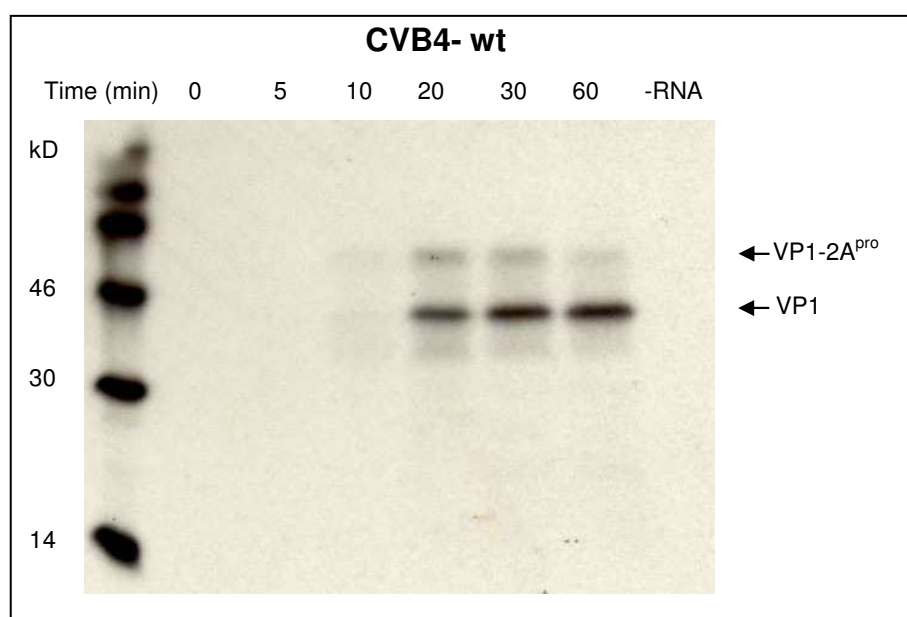


Fig.20: Self- processing of CVB4- VP1- 2A^{pro} during *in vitro* translation.

The mRNA CVB4-VP1-2A^{pro} was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} and the cleavage product VP1 are marked. After 20 min. 50% of VP1- 2A^{pro} are cleaved, after 60 min cleavage is almost completed.

Figure 21 demonstrates the translation of PV1 with the cleavage site of eIF4GI introduced. The 2A^{pro} was able to recognise the cleavage site of eIF4GI in *cis*. Cleavage started slightly delayed compared to the wild type after 30 minutes. However, after 180 minutes more than 50% were cleaved.

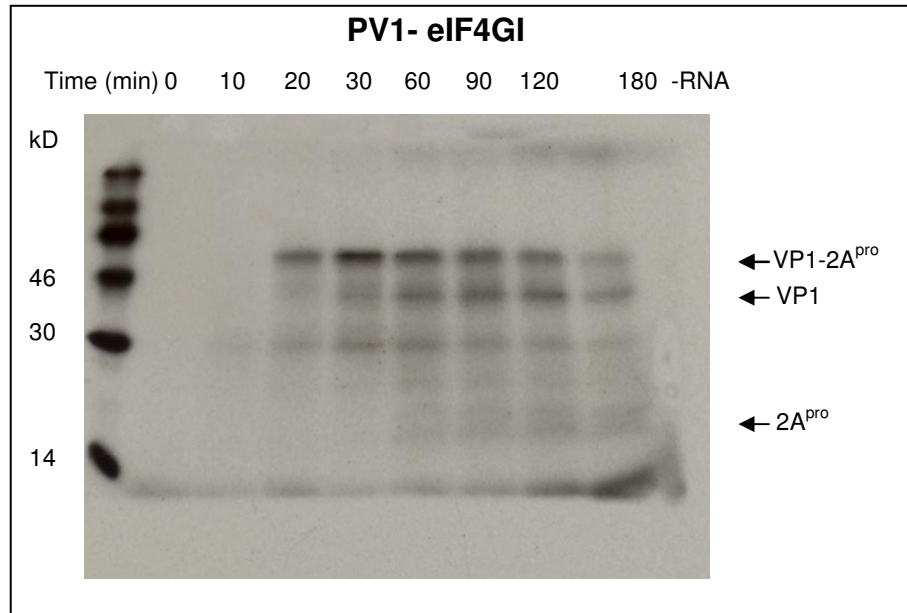


Fig.21: Self-processing of PV1-VP1-2A^{pro} with the cleavage site of eIF4GI inserted during *in vitro* translation. The cleavage of the polyprotein by 2A^{pro} was analysed as described in Fig.10. Uncleaved VP1-2A^{pro} and the cleavage products VP1 and 2A^{pro} are marked. Cleavage started after 30 min. and more than 50% were cleaved after 180 min.

In figure 22 it can be seen that the 2A^{pro} of CVB4 is also able to recognise eIF4GI in *cis*. It cleaves the mutant carrying eIF4GI with a similar efficiency to the wild type, with cleavage starting after 20 minutes and being almost complete after 60 minutes translation.

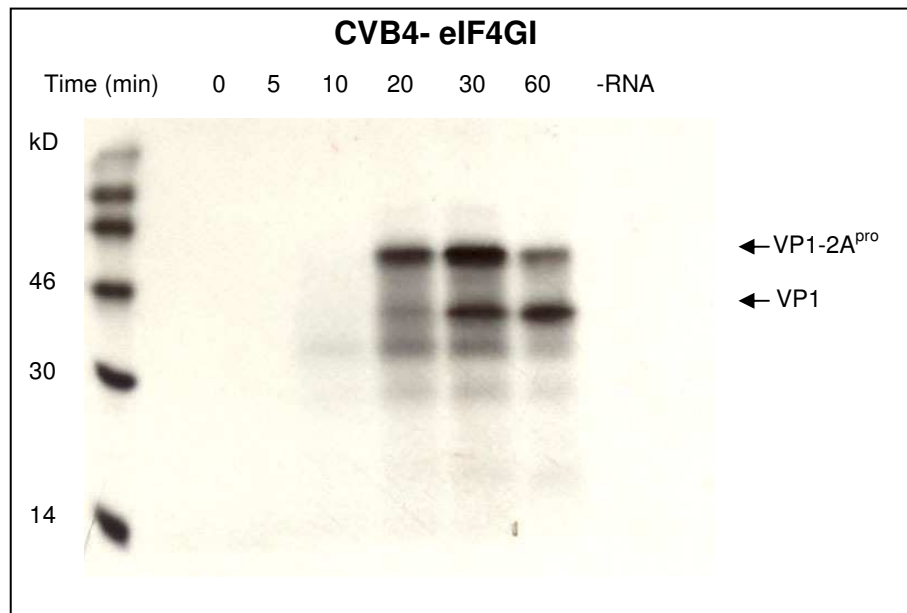


Fig.22: Self-processing during *in vitro* translation of CVB4-VP1- 2A^{pro} with the cleavage site of eIF4GI inserted. Analysis of the mRNA was performed as described in Fig.10. Uncleaved VP1-2A^{pro} and the cleavage product VP1 are marked. Cleavage started after 20 min. and was almost completed after 60 min.

5. DISCUSSION

5.1. Introduction of methionine at P1 position in HRV2 VP1 2A^{pro} and HRV14 VP1 2A^{pro}

As mentioned in the introduction, rhino- and enteroviruses are able to tolerate a wide variety of amino acids at the P1 position. Replacement of the alanine at the P1 of HRV2 by methionine has been shown to result in more efficient cleavage by 2A^{pro}. However, this data is only available for bacteria and synthetic peptide substrates, where the relative cleavage efficiency was shown to be fivefold higher than that of the wild type (Sommergruber *et al.*, 1992). This makes methionine an interesting target for the development of an inhibitor. It has been shown that zVAM.fmk, an inhibitor with methionine at P1, was more efficient in inhibiting intermolecular cleavage by HRV2 2A^{pro} than zVAD.fmk (Deszcz *et al.*, 2006). In this thesis, the cleavage of HRV2 and HRV14 with the mutation P1M was examined in a more sensitive system, RRLs.

A time course *in vitro* translation was used to assay the exact time points at which cleavage takes place. In wild type HRV2 VP1 2A^{pro}, the first bands are visible after 10min, after 20min 50% are cleaved and after 60min self-processing is completed. For the mutation HRV2 VP1 2A^{pro} P1M, a similar result could be observed. The first bands were observed after 20min 50% were cleaved and after 60min cleavage was completed. This indicates that self-processing in the mutant carrying the methionine instead of the P1 alanine works equally well as in the wild type. To find out whether cleavage of the mutant is more efficient than that of the wild type, further studies on the kinetics of the cleavage have to be done.

The results for HRV14 are quite similar to that of HRV2. The start of the self-processing of wild type HRV14 VP1 2A^{pro} is slightly delayed in comparison to that of HRV2 VP1 2A^{pro}. Cleavage starts after 20min, after 30min 50% are cleaved and after 60 min the self-processing is completed. Here, the mutant with the methionine at the P1 showed a slightly retarded cleavage compared to the wild type. Self-processing of HRV14 VP1 2A^{pro} P1M started after 20min, but was less than 50% completed after 30min and not completely finished after 60min. As in the case of HRV2, further studies would need to be done to obtain detailed information on the kinetics of the cleavage reaction.

These results show that the substitution of the alanine and the tyrosine at the P1 of HRV2 and HRV14, respectively, with methionine does not lead to a loss of function in self-processing even if the translation is tested in a sensitive system. This corresponds with the data in the literature for the self-processing in bacteria and with synthetic peptides and the successful use of the zVAM.fmk inhibitor (Sommergruber *et al.*, 1992; Deszcz *et al.*, 2006). However, it has to be said that no increase in the cleavage efficiency could be monitored, as this was the case for bacteria and synthetic peptide.

5.2. Insertion of the cleavage site of eIF4GII into HRV2 and HRV14

In addition to self-processing (Toyoda *et al.*, 1986), the 2A^{pro} of rhino- and enteroviruses also cleaves between the two isoforms of eIF4G after it has cleaved itself out of the polyprotein (Lloyd *et al.*, 1988; Gradi *et al.*, 1998). This cleavage is a crucial step in the viral life cycle as it leads to a shut-off of host cell translation (Kräusslich *et al.*, 1987; Lloyd *et al.*, 1987; Jewell *et al.*, 1990). The exact mechanism is described in chapter 1.2. This second cleavage of 2A^{pro} is performed in *trans* in contrast to the self-processing which is performed in *cis*. An interesting question is whether the sequences for this cleavage can also be recognised by 2A^{pro} in *cis* when they are inserted into the cleavage site between VP1 and 2A^{pro} on the polyprotein. This means finding out whether the amino acid sequence at the cleavage sites of the two isoforms of eIF4G are also recognised by 2A^{pro} when they are removed from their native protein surroundings and put in the polyprotein context. For HRV 2 and HRV14, this has already been examined for eIF4GI (Sousa *et al.*, 2006; Schmid, 2002). It has been shown that HRV2 2A^{pro} is able to recognise the cleavage site of eIF4GI in *cis* and self-processing was performed with kinetics that were similar to that of the wild type. On the other hand, the 2A^{pro} of HRV14 was not able to recognise eIF4GI in *cis* and no self-processing was observed. Further experiments demonstrated that HRV14 VP1 2A^{pro} cannot accept the arginine at the P1 position, found at P1 in eIF4GI. Substitution of the arginine at the P1 in eIF4GI by the tyrosine of wild type HRV14 VP1 2A^{pro} was sufficient to restore the self-processing activity but not to wild type levels.

In this thesis, a similar experimental set up has been used to test whether the 2A^{pro} is able to recognise the other isoform of eIF4G, eIF4GII, in *cis* when it is removed from its original protein context. Mutants were created, in which the amino acids IITTA * GPSD of the cleavage site of HRV2 VP1 2A^{pro} and DIKSY * GLGP of HRV14 VP1 2A^{pro}, respectively,

were replaced by the amino acid sequence PLLNV * GSRR of the cleavage site for 2A^{pro} on eIF4GII.

For the HRV2 mutant, no self-processing activity could be monitored during 180min of *in vitro* translation. This means that the 2A^{pro} was not able to recognise the cleavage site of eIF4GII in *cis*. For HRV14, very similar results were observed. Again, no self-processing activity was monitored, showing that HRV14 2A^{pro} was not able to recognise the eIF4GII sequence in *cis*. As has been shown for eIF4GI that the arginine at P1 could not be tolerated by the HRV 14 2A^{pro}, it was assumed that the valine at the P1 position of eIF4GII would also not be tolerated. Therefore, the valine at P1 of eIF4GII was substituted by the tyrosine of the wild type. The experiments showed that the replacement of the valine at P1 by tyrosine was sufficient to regain self-processing activity. The *cis* cleavage of eIF4GII started after 60min and was completed after 180min. However, the delay of the start of self-processing in comparison to the wild type indicates that other residues are involved in the recognition of the P1 residue. As HRV2 and HRV14 2A^{pro} share only 40% amino acid sequence identity it will only be possible to identify such residues when the crystal structure of HRV14 2A^{pro} becomes available. Furthermore, it would be interesting to examine whether the self-processing activity of HRV2 2A^{pro} as well is regained if the valine at the P1 of eIF4GII is substituted by the alanine of the wild type.

5.3. Insertion of the cleavage site of eIF4GI into PV1 and CVB4

As discussed in 5.2, it was examined whether the 2A^{pro} of HRV2 and HRV14 are able to recognise the cleavage sites of eIF4G in *cis* when they are removed from their native protein surroundings. This showed that the specificity of HRV2 2A^{pro} and HRV14 2A^{pro} differ significantly at P1.

Therefore, it is of interest to compare the substrate binding of HRV14 2A^{pro} to that of poliovirus 1 and coxsackievirus B4, which are both related to HRV14. It has to be remarked that CVB4 and PV1 2A^{pro} share about 58% sequence identity, which means that they are even more closely related than HRV2 and HRV14 2A^{pro} (Jenkins *et al.*, 1987).

During infection HRV14 and PV1 both cleave eIF4GI more rapidly than eIF4GII, which indicates that the respective 2A^{pro} poorly recognises the eIF4GII cleavage site in comparison to that of eIF4GI (Gradi *et al.*, 1998; Svitkin *et al.*, 1999). HRV2 2A^{pro} on the other hand cleaves eIF4GI and eIF4GII equally well during infection (Seipelt *et al.*, 2000).

It was examined in this thesis whether the 2A^{pro} of CVB4 and PV1 were able to recognise the cleavage site of eIF4GI in *cis* or whether self-processing activity would be lost as this is the case for HRV14 2A^{pro}.

It was reported in the results that CVB4 2A^{pro} was still able to recognise the cleavage site of eIF4GI in *cis* and self-processing was maintained to almost wild type level. When the same experiment was repeated for PV1, the mutant that carried the cleavage site of eIF4GI self-processing was also maintained, although slightly delayed compared to the wild type. The experiments showed that PV1 and CVB4 were able to recognise the cleavage site of eIF4GI in *cis*.

It has been shown for HRV2 and HRV14 2A^{pro} that the amino acid at P1 position plays a major role in *cis* processing of eIF4GI (Sousa *et al.*, 2006). As discussed in 5.2, HRV14 2A^{pro} could not accept the arginine at P1 instead of the wild type tyrosine. PV1 on the other hand, which carries a tyrosine at the P1 position as well, was able to tolerate arginine at P1. Self-processing was maintained in the mutant although delayed compared to the wild type.

Therefore, it is of interest to examine which residues are involved in the recognition of the P1 amino acid. The crystal structure of HRV2 2A^{pro} revealed that the side chain of residue C101 protrudes into the S1 pocket on the enzyme recognising the P1 (Petersen *et al.*, 1999). In the pocket the negatively charged side chain of E102 probably helps accepting the positive charge of the P1 arginine of eIF4GI. For the HRV14 2A^{pro} residue A104, which is the equivalent to HRV2 2A^{pro} C101, a similar role in substrate recognition has been revealed (Sousa *et al.*, 2006). This indicates that amino acid positions equivalent to residue 101 in HRV2 2A^{pro} are also involved in the substrate recognition of other picornaviral 2A proteases.

In poliovirus 1 2A^{pro} a tyrosine is found at P1 and an alanine at residue 104, which is equivalent to HRV2 2A^{pro} residue 101. As the same residues as in PV1 are found in HRV14 2A^{pro} it is surprising that PV1 2A^{pro} was able to cleave eIF4GI in *cis*, although delayed to the wild type, while HRV14 2A^{pro} was not. This indicates that other residues are involved in the recognition of the P1 residue as well. It has to be remarked that it is still not determined whether the cleavage site of HRV14 on eIF4GI is identical to that used by HRV2, PV1 and CVB4 (Lamphear *et al.*, 1993; Zamora *et al.*, 2002) which might be another explanation for the differences between HRV14 and PV1 *cis* cleavage of eIF4GI. Therefore, it will be important to identify this cleavage site for HRV14.

5.4. Insertion of the cleavage site of HRV14 into HRV2, the one of HRV2 into HRV14

HRV2 and HRV14 are two out of one hundred serotypes of human rhinovirus (Rueckert *et al.*, 1996). They differ to a large extent. Their amino acid sequence shows overall only 51% identity, the sequences of their 2A proteases are only 40% identical (Wang *et al.*, 1998). The 2A^{pro} of HRV14 is more closely related to that of CVB4 and PV1. Within the group of rhinoviruses, they are divided into different groups. HRV2 is classified as a group A rhinovirus and belongs to the minor group, which means that it uses LDL- receptors. HRV14 on the other hand is classified as a group B rhinovirus of the major group, which means it uses ICAM- receptors (Savolainen *et al.*, 2002b; Duechler *et al.*, 1993). Due to the differences in their sequences it is difficult to gain information for HRV14 from the crystal structure of HRV2 (Petersen *et al.*, 1999). Although the catalytical triad is identical for both, the sequence of HRV14 differs from that of HRV2 in several important regions. Most important, the cleavage for self-processing is IITTA * GPSD for HRV2 2A^{pro}, that of HRV14 2A^{pro} is DIKSY * GFGH: At residue 101, which plays an important role in substrate recognition, as discussed in 5.3, a cysteine is found in HRV2. At the equivalent position in HRV14 an alanine is found. Still, their overall structures, the viral life cycles and the proteolytical activity of 2A^{pro} are similar. While different cleavage sites are recognised by HRV2 2A^{pro} and HRV14 2A^{pro} during self-processing, both 2A proteases are able to recognise and cleave eIF4GI and eIF4GII.

Therefore it was of interest whether the 2A^{pro} of HRV2 would be able to recognise the cleavage site of HRV14 and *vice versa* when they are introduced between VP1 and 2A. We showed here that the 2A^{pro} of HRV2 is not able to recognise the cleavage site of HRV14 in *cis*. It was shown that HRV2 2A^{pro} can accept a tyrosine, as present in HRV14 at P1 position without significant impairment of self-processing. Substitution of the P' region of HRV2 by that of HRV14 on the other hand proved to be deleterious (Skern *et al.*, 1990). Therefore, the residues at the P' region are likely to be responsible for the loss of self-processing activity that was monitored after insertion of the HRV14 cleavage site. Although the P' region is highly conserved in rhinoviral 2A proteases, that of HRV14 differs significantly from the other 2A^{pro}. At P1' in all serotypes a glycine is found, mutations replacing that amino acid are deleterious in all cases (Glaser & Skern, 2000). While this is also true for HRV14, at P2' in HRV14 a leucine is found, where all other rhinoviral 2A^{pro} have a proline. It has been shown that substitution of the proline at P2' eliminated cleavage (Skern *et al.*, 1990). The crystal structure of HRV2 2A^{pro} (Petersen *et al.*, 1999) revealed that the proline sticks out of the

active site. The leucine found in HRV14 at this position is a large hydrophobic residue which probably can not be recognised by HRV2 2A^{pro}. Furthermore, HRV14 also differs significantly from HRV2 2A^{pro} at the P2 position. In HRV14, serine is present, while in all other rhino- and enteroviruses threonine or asparagine are found. They might also interfere with substrate recognition by HRV2. Creation of further deletion mutants could be used to determine whether other residues are involved as well.

In contrast to HRV2, HRV14 2A^{pro} did recognise the cleavage site of HRV2 in *cis* and was still able to cleave itself from the polyprotein. It has to be remarked that self-processing of the mutant was significantly slower than that of wild type HRV14. Previous work on the HRV14 substrate specificity was limited to synthetic peptides. It was shown that HRV14 2A^{pro} poorly cleaves a peptide corresponding to its wild type cleavage site *in vitro*. Cleavage was improved when the peptide resembled more the HRV2 2A^{pro} cleavage site (Wang *et al.*, 1998b). This data shed no light on the P1 specificity as the P1 position of the peptides examined was always tyrosine as in the wild type. Recently it was revealed that HRV14 can not accept an arginine at P1, as its found in eIF4GI (Sousa *et al.*, 2006). It would be interesting to replace the tyrosine at P1 of HRV14 2A^{pro} by the alanine found in HRV2 2A^{pro} to find out whether this residue is responsible for the retarded self-processing of HRV14 after then insertion of the HRV2 cleavage site.

6. SUMMARY AND ZUSAMMENFASSUNG

6.1. Summary

This diploma thesis deals with the picornaviral 2A proteinases of two serotypes of human rhinovirus, HRV2 and HRV14, as well as one serotype of poliovirus, PV1, and coxsackievirus, CVB4. The cleavage specificities of these proteases were examined by monitoring the self-processing activity. Different mutants were created by cassette cloning and the effect of the mutations on the self-processing was investigated.

First, it was examined whether a substitution of the amino acid at the P1 position of HRV2 and HRV14 2A^{pro} by a methionine would enhance *in vitro* translation in rabbit reticulocyte lysate (RRL), as it does in bacteria and on synthetic peptide substrates. We show here that *in vitro* translation in RRLs of a mutation of HRV2 2A^{pro} with methionine at P1 works as well as that of the wild type, but does not enhance it. For HRV14 2A^{pro} P1M cleavage was even slightly retarded compared to the wild type.

The next step was to find out whether the 2A^{pro} of HRV2 and HRV14 was able to cleave the eIF4GII cleavage site in *cis* when embedded in the polyprotein. The same experiment has already been done with the sequence of the cleavage site of eIF4GI, showing that HRV2 2A^{pro} can recognise eIF4GI in *cis* while HRV14 2A^{pro} is not able to do so. After constructing the mutants and *in vitro* translating the corresponding RNA, we discovered that both respective 2A^{pro}s of HRV2 and HRV14 were not able to exert self-processing. This means that neither of the two proteases was able to cleave the eIF4GII cleavage site in *cis*.

Following this, we showed that substitution of the valine at P1 of the eIF4GII mutant by the tyrosine of the wild type was sufficient to regain self-processing activity in HRV14. Self-processing was not regained to wild type level, indicating that other residues are involved in the substrate recognition.

A similar experiment was then arranged for PV1 and CVB4 in which it was tested whether their 2A proteinases were able to cleave the eIF4GI cleavage site in *cis*. Our experiments showed that both PV1 and CVB4 2A^{pro} were able to cleave eIF4GI in *cis* and self-processing activity was sustained, although slightly delayed in comparison to the wild type. This is surprising as in PV1 a tyrosine is found at P1 position and an alanine is found on the residue equivalent to 101 in HRV2 2A^{pro}. As these residues are equivalent to HRV14, which is not able to cleave eIF4GI in *cis*, this indicates that either other residues are involved or the HRV14 might not have the same eIF4GI cleavage site as HRV2, PV1 and CVB4. CVB4

2A^{pro} additionally has a more polar surface than HRV2, so a different cleavage mechanism might be used.

Finally, it was observed whether the 2A^{pro} of HRV2 would be able to cleave the HRV14 VP1-2A cleavage site when embedded into the HRV2 polyprotein and *vice versa*. We discovered that HRV2 was not able to cleave HRV14 when embedded into its own polyprotein. Indeed, self-processing activity was completely lost. This is probably due to the P' region of HRV14 2A^{pro}, as it differs significantly from that found in all other rhinoviruses. Different results were observed for HRV14. The 2A^{pro} of HRV14 still was able to cleave itself out of the mutated polyprotein. It has to be remarked that self-processing started significantly delayed in comparison to the wild type.

6.2. Zusammenfassung

Die vorliegende Diplomarbeit beschäftigt sich mit den picornaviralen 2A Proteasen zweier Serotypen des humanen Rhinovirus, HRV2 und HRV14, sowie mit jeweils einem Serotyp der Polioviren, PV1, und der Coxsackieviren, CVB4. Die Spezifität des Schnittverhaltens dieser Proteasen wird anhand der Selbstprozessierungsaktivität untersucht. Verschiedene Mutanten wurden mittels Kassettenklonierung hergestellt und die Auswirkungen der Mutationen auf die Selbstprozessierung wurden untersucht.

Zunächst wurde untersucht, ob eine Substitution der Aminosäure an der P1 Position von HRV2 oder HRV14 durch ein Methionin in der Lage ist, die *in vitro* Translation in RRL zu beschleunigen, wie das bereits in Bakterien und für synthetische Peptide gezeigt wurde. In dieser Arbeit stellten wir fest, dass die *in vitro* Translation der HRV2 2A^{pro} Mutante mit dem Methionin an der P1 Position in RRL so effektiv abläuft wie für den Wildtyp, jedoch nicht beschleunigt ist. Für HRV14 2A^{pro} P1M zeigte sich sogar ein leicht verzögertes Schnittverhalten.

Der nächste Schritt war es, herauszufinden ob die 2A^{pro} von HRV2 und HRV14 in der Lage ist, die Schnittsequenz von eIF4GII in *cis* zu erkennen, wenn diese in das Polyprotein eingebettet ist. Ein ähnliches Experiment wurde bereits mit der Schnittsequenz von eIF4GI durchgeführt, die ebenfalls in das Polyprotein eingebaut worden war. Diese Experimente zeigten, dass die 2A^{pro} von HRV2 in der Lage war, die Schnittsequenz in *cis* zu erkennen, während die 2A^{pro} von HRV14 dies nicht konnte. Nach Konstruktion der Mutanten und *in vitro* Translation der entsprechenden RNA fanden wir heraus, dass die jeweiligen 2A Proteasen von HRV2 und HRV14 nicht in der Lage waren, die Schnittsequenz von eIF4GII in *cis* zu erkennen.

Anschließend zeigten wir, dass die Substitution des Valins an der P1 Position der eIF4GII Mutante durch das Tyrosin des Wildtyps ausreichend war um die Selbstprozessierung von HRV14 VP12A wieder zu aktivieren. Es muss jedoch erwähnt werden, dass der Grad der Selbstprozessierung des Wildtyps nicht wiederhergestellt werden konnte, was nahe legt, dass zusätzlich andere Reste an der Substraterkennung beteiligt sind.

Ein ähnliches Experiment wurde dann für PV1 und CVB4 durchgeführt. In diesem wurde untersucht, ob deren 2A Proteasen die Schnittsequenz von EIF4GI in *cis* erkennen können. Unsere Experimente zeigten, dass sowohl die PV1 als auch die CVB4 2A Protease eIF4GI in *cis* erkennen konnten und die Selbstprozessierung aufrechterhalten werden konnte, auch wenn sie, im Vergleich mit dem Wildtyp, leicht verzögert stattfand. Das ist überraschend, da an P1 in PV1 2A^{pro} ein Tyrosin vorliegt und die Position äquivalent zu 101 in HRV2 mit Alanin

besetzt ist. Das entspricht der Besetzung dieser Reste in HRV14 2A^{pro}, das eIF4GI in *cis* nicht erkennen kann. Daraus lässt sich schließen, dass entweder zusätzlich andere Reste an der Substraterkennung beteiligt sind oder HRV14 eIF4GI vielleicht an einer anderen Stelle schneidet als PV1, HRV2 und CVB4. CVB4 2A^{pro} besitzt darüber hinaus eine viel polarere Oberfläche als HRV2, was möglicherweise auf die Benutzung eines anderen Mechanismus hinweist.

Abschließend wurde untersucht, ob die 2A Protease von HRV2 in der Lage ist, die Schnittsequenz von HRV14 zu erkennen, wenn diese in das HRV2 Polyprotein eingebettet ist. Das gleiche Experiment wurde umgekehrt auch für HRV14 durchgeführt. Unsere Experimente zeigten, dass HRV2 die Schnittsequenz von HRV14 auf dem eigenen Polyprotein nicht erkennen und schneiden konnte, es war keinerlei Selbstprozessierungsaktivität zu beobachten. Das liegt vermutlich an der P' Region von HRV14 2A^{pro}, die sich deutlich von der aller anderen Rhinoviren unterscheidet. Für HRV14 zeigten sich jedoch andere Ergebnisse: Die 2A Protease war in der Lage, die Schnittsequenz von HRV2 eingebettet in das eigene Polyprotein zu erkennen und sich aus dem mutierten Polyprotein herauszuschneiden. Es muss jedoch erwähnt werden, dass auch hier nicht die Geschwindigkeit der Selbstprozessierung des Wildtyps erreicht werden konnte.

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Curriculum vitae

Personal Data

Name: Stephanie Schertler

Date of birth: 13.03.1982

Education:

09/1988- 07/1992 Volksschule Schäffergasse 1040 Vienna

09/1992- 06/2000 BRG IV Waltergasse, Vienna

10/2000- 09/ 2008 Undergraduate study in chemistry, with emphasis on biochemistry

07/2007- 08/2008 Diploma thesis

Employment:

07/2001- 03/2006: Part time position at NUA- Umweltanalytik (Maria Enzersdorf) as analytical chemist for waste and waste water analysis

Languages:

German (native language)

English (fluent)

Italian (intermediate knowledge)

Russian (lower intermediate knowledge)

Spanish (basic knowledge)