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Abstract / Zusammenfassung

Foot-and-mouth disease virus (FMDV) is a member of the picornavirus family, consisting of small non-enveloped icosahedral viruses with an RNA genome of positive sense. The family contains twelve different genera, including important human and animal pathogens. The RNA genome encodes one large open reading frame from which a single polyprotein is translated. This single polyprotein is processed by viral proteases resulting in mature viral proteins.

The papain-like leader protease (Lb^{pro}) of FMDV performs the initial cleavage on the polyprotein between its own C-terminus and the N-terminus of the structural protein VP4. This cleavage has been shown to be favored in cis (intramolecular), although also trans (intermolecular) cleavage is observed *in vitro*. However, intramolecular processing is preferred in vivo. The only other substrates cleaved by Lb^{pro} besides the viral polyprotein are the two homologues of the host cell eukaryotic translation initiation factor 4G (eIF4GI and eIF4GII). eIF4G is crucial for translation initiation acting as a scaffold and bringing the components of the translation initiation complex in close proximity. Once eIF4G is cleaved by Lb^{pro} , it loses this property. Consequently, the protein synthesis of the cell is inhibited as the recruitment of capped mRNAs to the 40S ribosomal subunit is no longer possible. However, viral translation is unaffected as it initiates via an internal ribosomal entry segment (IRES) present on the 5' untranslated region of the viral genome. Therefore, the virus is still able to exploit the host cell translation machinery.

Lb^{pro} is a cysteine protease with a very narrow substrate specificity mainly determined by the S_2 pocket. Only leucine, valine and, to a certain extent, isoleucine are accepted at this site. The cleaved sequences on the viral polyprotein and eIF4GI both contain leucine at S_2 , whereas valine occupies this position in eIF4GII. Despite these constraints, no consensus sequence can be determined as the sites differ strongly from each other. Recently, it has been shown that especially one residue involved in the formation of the hydrophobic S_2 pocket is of great importance for the maintenance of the proteases specificity. Molecular modeling and mutational analysis demonstrated that Leu143 is able to discriminate a substrate containing phenylalanine at the P_2 position. Thus, a Leu143Ala variant of Lb^{pro} was able to accept phenylalanine. Therefore, sterical hindrances caused by leucine might be the reason for this discrimination. The only other residue found at position 143 in Lb^{pro} of different FMDV strains is methionine. Hence, the presence of either leucine or methionine at position 143 of Lb^{pro} is crucial for the high

specificity of the protease. Furthermore, the ability to cleave substrates containing phenylalanine at P₂ seems to be of biological relevance for the virus.

This thesis compares the substrate specificity of Lb^{pro} wt with the altered specificity of Lb^{pro} L143A. To demonstrate the change in specificity, an eIF4GII fragment containing the Lb^{pro} and eIF4E binding sequence was used as substrate. As expected, Lb^{pro} wt generated only two cleavage products whereas Lb^{pro} L143A produced additional cleavage products. A sequence related to the cleavage sequence of Lb^{pro} on eIF4GII is not recognized by Lb^{pro} wt due to the presence of aspartic acid at P₃ and phenylalanine at P₂. As Lb^{pro} L143A was shown to accept phenylalanine at P₂, we speculated that this related sequence might be cleaved as well. However, mass spectrometry analysis of two aberrant cleavage products gave no explicit indication that this sequence is indeed recognized.

Further investigations on the specificity included the eukaryotic translation initiation factor 4E (eIF4E). eIF4E binds capped cellular mRNAs and the N-terminal region of eIF4G. Previous experiments demonstrated that upon binding of eIF4E, eIF4G undergoes an unfolded-to-folded transition. As a result, the eIF4G cleavage sequence is exposed and becomes better accessible for Lb^{pro} enhancing the cleavage efficiency. However, in our *in vitro* approach adding different amounts of eIF4E to eIF4GII prior to cleavage did not change the efficiency of cleavage by Lb^{pro} wt or Lb^{pro} L143A.

In a last set of experiments, a second substrate, eIF4GII^{self}, was used. In eIF4GII^{self} the natural cleavage sequence is exchanged with the Lb^{pro} self-processing site. Despite the different chemical environment in eIF4GII than found in the viral polyprotein, Lb^{pro} wt and Lb^{pro} L143A were able to process eIF4GII^{self}. Furthermore, a lower amount of protease was needed to process 50% of eIF4GII^{self} compared to eIF4GII. These findings suggest that the self-processing sequence is, in spite of the changed chemical environment better recognized by Lb^{pro} than the natural sequence of eIF4GII.

Der Maul-und-Klauenseuche-Virus (FMDV) gehört zur Familie der Picornaviridae. Diese umfasst kleine Viren ohne Lipidhülle, mit ikosaedrischem Kapsid sowie einem Genom positiver Polarität. Die Familie der Picornaviridae kann in zwölf verschiedene Genera unterteilt werden, welche sowohl für den Menschen als auch für Tiere wichtige Pathogene enthalten. Das RNA Genom besteht aus einem offenen Leserahmen von dem ein einziges Polyprotein abgelesen wird. Dieses wird anschließend von viralen Proteasen prozessiert.

Die Papain-ähnliche Leader Protease (Lb^{pro}) des Maul-und-Klauenseuche-Virus spaltet das Polyprotein zunächst zwischen dem eigenen C-terminus und dem N-terminus des strukturellen Proteins VP4. Es wurde gezeigt, dass diese Reaktion in cis (intramolekular) bevorzugt wird allerdings auch in trans (intermolekular) stattfinden kann. *In vivo* wird allerdings die intramolekulare Reaktion favorisiert. Neben dem viralen Polyprotein spaltet Lb^{pro} die zwei Homologen des eukaryotischen Initiationsfaktor 4G (eIF4GI und eIF4GII) der Wirtszelle. eIF4G spielt eine zentrale Rolle während der Translationsinitiation und sorgt als Verbindungsprotein für den Zusammenhalt der einzelnen Komponenten des Translationsinitiationskomplexes. Sobald eIF4G von Lb^{pro} gespalten wird, zerfällt dieser Komplex und die Wirtszelle kann keine gecappte mRNA zur 40S Untereinheit rekrutieren. Dadurch wird die Proteinsynthese der Wirtszelle verhindert. Die virale Translation hingegen ist nicht betroffen, da der Virus über ein sogenanntes internes ribosomales Eintrittssegment (IRES) initiiert. Infolgedessen übernimmt der Virus die Kontrolle über die Translationsmaschine der Wirtszelle.

Lb^{pro} ist eine Cysteinprotease mit einer stark eingeschränkten Substratspezifität, welche hauptsächlich von der S_2 Tasche bestimmt wird. Ausschließlich Leucin, Valin sowie in gewissem Ausmaß werden an dieser Stelle akzeptiert. Die Sequenz bei der Lb^{pro} das viral Polyprotein und eIF4GI spaltet enthält Leucin an dieser Stelle, wohingegen Valin diese Position in eIF4GII besetzt. Eine Konsensussequenz kann trotz dieser Übereinstimmung nicht festgelegt werden, da die einzelnen Sequenzen zu stark variieren. Vor Kurzem wurde gezeigt, dass eine weitere Aminosäure der hydrophobischen S_2 Tasche äußerst wichtig ist für die Aufrechterhaltung der Spezifität von Lb^{pro} . Molekulardesign und Mutationsanalysen ergaben, dass Leu143 ein wichtiger Faktor ist um Substrate welche Phenylalanin an Position P_2 enthalten auszuschließen. Die Mutation Leu143Ala ermöglichte hingegen das Eindringen von Phenylalanin in die S_2 Tasche. Folglich dürften sterische Hinderungen der Grund für die Diskriminierung von Phenylalanin sein. Die einzig andere Aminosäure die an der Position 143 in anderen FMDV Stämmen gefunden wurde ist nur Methionin. Demnach sind Leucin oder Methionin an Position 143 von biologischer Relevanz um jegliche Substrate mit Phe an Position P_2 auszuschließen.

Diese Diplomarbeit vergleicht die Substratspezifität des Wildtyps (Lb^{pro} wt) mit der veränderten Spezifität von Lb^{pro} L143A. Um den Unterschied aufzuzeigen wurde ein eIF4GII Fragment als Substrat verwendet. Dieses enthält sowohl die Lb^{pro} Spaltsequenz als auch die eIF4E Bindungssequenz. Erwartungsgemäß wurden mit Lb^{pro} wt nur zwei Spaltprodukte erhalten, wohingegen Lb^{pro} L143A zusätzliche Spaltprodukte produziert. Eine Sequenz ähnlich jener die Lb^{pro} in eIF4GII erkennt, wird allerdings nicht von Lb^{pro} wt gespalten. Diese verwandte Sequenz wird aufgrund von Asp an Position P₃ und Phe an Position P₂ nicht erkannt. Da Lb^{pro} L143A Phe an Position P₂ akzeptieren kann, vermuteten wir, dass auch die ähnliche Sequenz geschnitten wird. Mittels Massenspektrometrie wurden zwei abweichende Spaltprodukte untersucht. Allerdings konnte nicht eindeutig bewiesen werden, dass Lb^{pro} L143A diese Sequenz erkennt.

Desweiteren wurde bei späteren Experimenten zusätzlich der eukaryotische Initiationsfaktor 4E (eIF4E) eingesetzt. eIF4E bindet einerseits zelluläre gecappte mRNAs, andererseits aber auch die N-terminale Region von eIF4G. Vorangehende Experimente haben gezeigt, dass eIF4G von einer ungeordneten in eine geordnete Konformation übergeht sobald es an eIF4E bindet. Dadurch wird die Region der Spaltung vermutlich stärker exponiert und ist für Lb^{pro} leichter zugänglich. Dies wiederum sollte zu einer verbesserten Prozessierung durch Lb^{pro} führen. Allerdings konnte bei meinem *in vitro* Versuchen keine Effizienzsteigerung durch den Zusatz von eIF4E zu eIF4G und anschließender Zugabe der Protease beobachtet werden.

Abgesehen von eIF4GII wurde ein weiteres Substrat verwendet, nämlich eIF4GII^{self}. eIF4GII^{self} enthält die Selbstprozessierungssequenz von Lb^{pro} statt der natürlichen Spaltungssequenz. Obwohl die chemische Umgebung in eIF4GII eine andere ist als jene im viralen Polyprotein, schneiden beide Proteasen (Lb^{pro} wt und Lb^{pro} L143A) die Selbstprozessierungssequenz. Überdies werden 50% eIF4GII^{self} bereits bei einer geringeren Menge an Protease geschnitten als eIF4GII. Folgedessen könnte man annehmen, dass Lb^{pro} die Selbstprozessierungssequenz mit größerer Effizienz erkannt wird als die natürliche Sequenz in eIF4GII.

1 Introduction

1.1 Picornaviruses

The family of *Picornaviridae* belongs to the order of Picornavirales and consists of twelve different genera. Viruses of these twelve genera infect vertebrates and include important human and animal pathogens such as hepatitis A virus (HAV), human rhinovirus (HRV), poliovirus (PV) and foot-and-mouth disease virus (FMDV) (ICTV 2009).

Picornaviruses are non-enveloped, single stranded RNA viruses of positive sense. The virions are spherical and about 30 nm in diameter. The particles are constructed in a simple way, consisting of an icosahedral protein shell which surrounds the naked RNA genome (Fields *et al.* 2007).

1.2 Foot-and mouth disease virus

Foot-and-mouth disease virus (FMDV) together with bovine rhinitis B virus and equine rhinitis A virus constitute the genus aphthovirus of the picornavirus family (ICTV 2009). Seven serotypes (A, O, C, Asia 1 and South African Territories 1, 2 and 3) of FMDV have been identified, each occurring in several subtypes. FMDV is the causative agent of foot-and-mouth disease, a highly infectious viral disease affecting wild and domestic cloven-hoofed animals such as cattle, swine and sheep (Fenner *et al.* 1993). FMDV is transmitted via direct contact between animals, by animal products (milk, meat and semen) and via the airborne route. The symptoms include vesicular lesions on tongue, feet, snout and teats, fever and lameness. The mortality rates in adult animals are quite low, whereas in young animals the virus affects the heart and may lead to higher lethality.

Foot-and-mouth disease was first described in the 16th century (Fracastorius 1546). 400 years later it was the first animal pathogen identified as a virus (Loeffler *et al.* 1897) and is therefore, besides poliovirus, one of the best-studied viruses. Major outbreaks of FMDV have occurred in nearly all livestock-containing regions except New Zealand and always caused great expense.

1.3 The virion

1.3.1 Genome organization

The genetic information of picornaviruses is encoded on a single-stranded RNA genome of positive polarity, containing only one open reading frame (ORF) (Fields *et al.* 2007). The FMDV genome contains untranslated regions (UTRs) downstream and upstream of the ORF.

The 5'-UTR, presented in figure 1, is about 1.300 bases and highly structured (Belsham *et al.* 2004). It contains five different elements involved in viral translation and RNA replication. The S-fragment is the most 5' element and folds into a stem-loop. Its function in the FMDV genome has not yet been determined. However, comparisons with other picornaviruses imply that it might be involved in genome stability in infected cells (Barton *et al.* 2001) and in the binding of proteins during genome replication (Andino *et al.* 1990, Andino *et al.* 1993, Herold *et al.* 2001). A poly(C) tract of about 100 bases follows the S-segment. The length of this element can vary strongly, but its exact function is still unknown (Rieder *et al.* 1993). Downstream of the poly(C) tract lies a series of RNA pseudoknots of unknown function (Escarmis *et al.* 1995) followed by a hairpin loop structure, the cis-acting replicative element (*cre*) (Mason *et al.* 2002). The *cre* element consists of a stem loop containing a conserved AAACA sequence and is crucial for RNA genome replication. The *cre* is located within different regions of the ORF in other picornavirus species, whereas in FMDV it is found in the 5' UTR. Between the *cre* and the ORF region, a series of highly conserved stem loops build the internal ribosome entry site (IRES). Picornaviral mRNAs lack the 7-methylguanosine cap structure, present in most eukaryotic mRNAs. Thus, ribosomes enter the genome internally at the IRES (Niepmann *et al.* 1997). The IRES region of aphthoviruses is about 450 nucleotides in length controlling the translation of the viral RNA (Macejak *et al.* 1991, Belsham *et al.* 1990, Kuhn *et al.* 1990). One of the five domains of the IRES (Pilipenko *et al.* 1992b) is a pyrimidine-rich region located at its 3' end, directly before the AUG initiation codon. At the very 5' end, the viral protein VPg (viral protein, genome linked) is covalently bound to the picornaviral RNA genome (Paul *et al.* 2003) and possibly primes RNA synthesis (Gerber *et al.* 2001).

The 3'-UTR, which follows the ORF, is much shorter and contains a stretch of RNA forming stem-loop structures (Pilipenko *et al.* 1992a) followed by a poly(A) tail (Serrano *et al.* 2006). The 3' UTR may be essential for genome replication (Melchers *et al.* 1997, Pilipenko *et al.* 1996, Rohll *et al.* 1995). This hypothesis is supported by the fact that several picornaviral proteins involved in RNA replication bind to this region (Cui *et al.* 1993, Cui *et al.* 1995, Harris *et al.* 1994). Furthermore, studies demonstrated that deletion of the FMDV 3' UTR reduces efficiency of the viral *in vitro* translation (Lopez de Quinto *et al.* 2002). The poly(A) tail is thought to be crucial for translation as well as RNA replication (Barton *et al.* 2001, Herold *et al.* 2001).

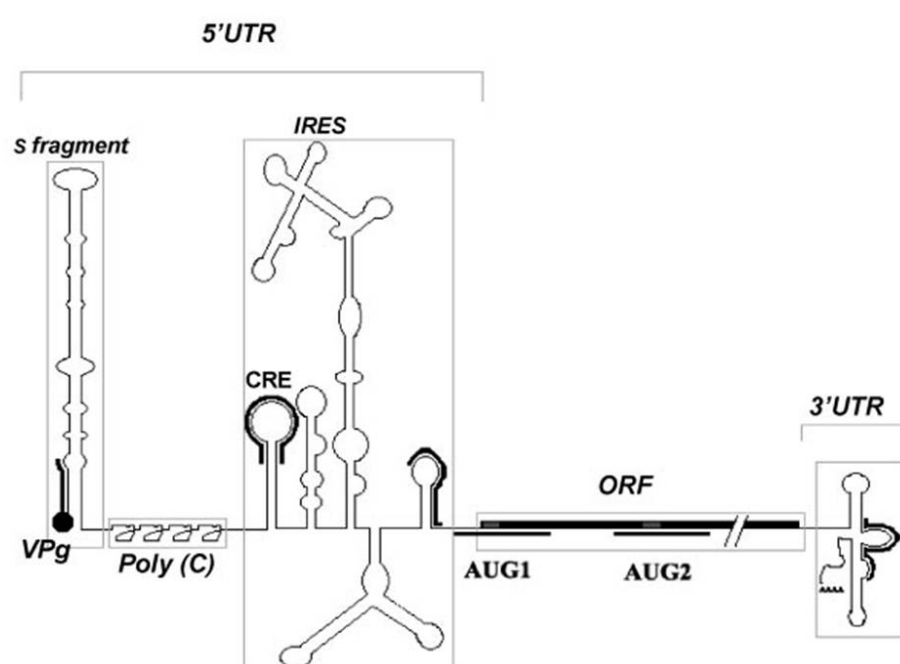


Figure 1: Schematic representation of the FMDV RNA genome focusing on the 5' and 3' UTRs. (Vagnozzi *et al.* 2007)

The single ORF itself is organized in four regions, L and P1-P3 (figure 2). The L region encodes two alternative AUG initiation codons. Therefore, the translation results in two different forms of the L protein, Lab and Lb (Carrillo *et al.* 2005). Lab (20 kDa) being the protein produced by initiation from the first AUG codon, and Lb (16 kDa) from the second AUG. Data suggests that Lb is the biologically functional protein *in vivo* (Piccone *et al.* 1995).

The L region is followed by the P1 region encoding the four structural proteins, viral protein 1 (VP1)-VP4. The P2 and P3 region, on the contrary, code for non-structural proteins. P2 is coding for 2A, 2B and 2C. In FMDV the 2A protein has no proteolytic activity in contrast to other picornavirus species such as HRV and PV (Donnelly *et al.*

2001). However, a separation event occurs at the N-terminus of the 2A protein and the C-terminus of VP1 which is due to a proposed ribosomal skip (de Felipe *et al.* 2003). P3 encodes 3A, 3B, 3C^{pro} and 3D^{pol}. 3C^{pro} and its precursor 3CD^{pro} have been identified as viral proteases (Birtley *et al.* 2005) that process the viral polyprotein. However, 3D^{pol} is a RNA-dependent RNA polymerase (Ferrer-Orta *et al.* 2003).

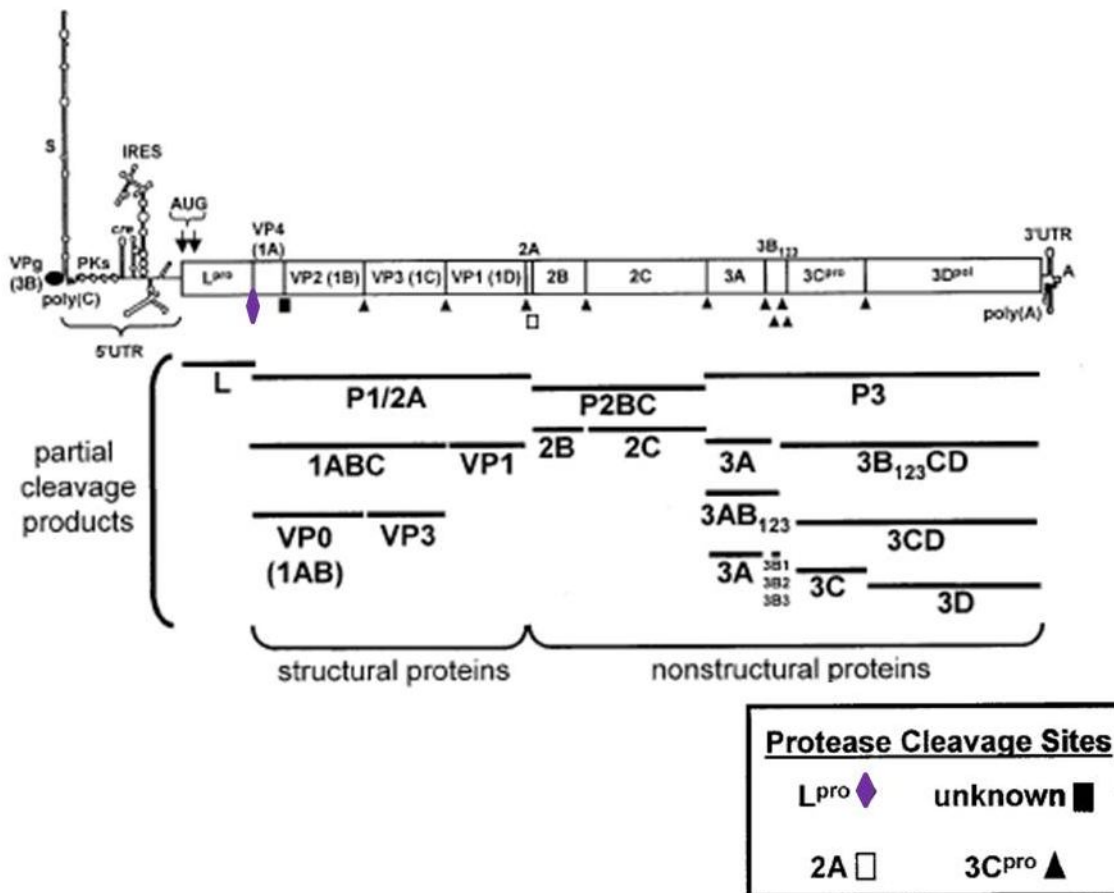


Figure 2: Organization of the FMDV RNA genome. The open boxes indicate the ORF and the viral proteins. The partial cleavage products are shown at the bottom. RNA structures of the 5' and 3' UTRs are shown as lines (adapted from Mason *et al.* 2003).

1.3.2 Capsid organization

The picornaviral capsid consists of 60 protomers that are tightly packed to form an icosahedron (figure 3a). Each protomer contains one copy of viral protein 1 (VP1) - VP4 (figure 3b). VP1, VP2 and VP3 build the outer surface whereas VP4 is located on the inner surface of the capsid (Fields *et al.* 2007). Even though the structural proteins VP1-VP3 have no sequence homology, they share the same topology. All three fold into an eight-stranded wedge-shaped β -barrel (Burke *et al.* 1991). The strands of the β -barrel are connected by loops.

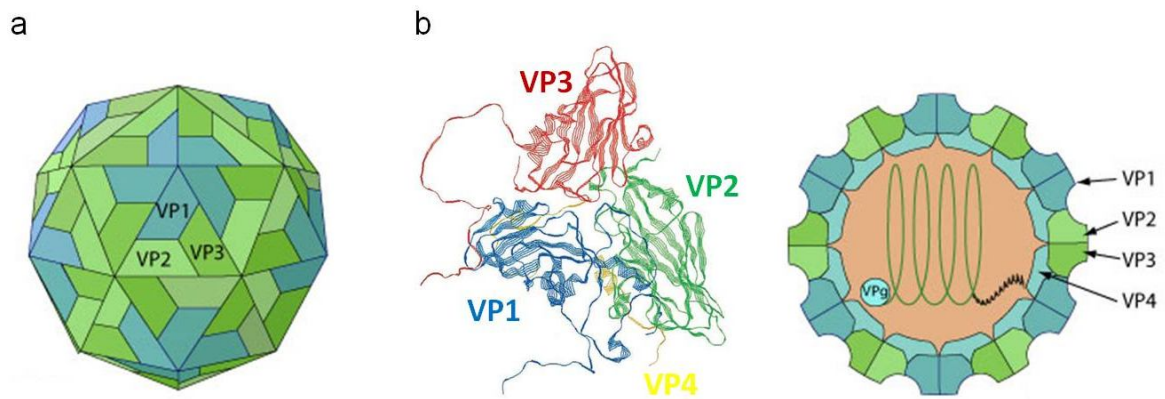


Figure 3: Structure of the picornaviral protomer and capsid. (a) Schematic view of an icosahedral capsid, showing the organization of the protomers (expasy.org). (b) Structure of the mature type O₁BFS FMD virion based on X-ray crystallography. A viral protomer showing β -barrel-and-loop organization of VP1-VP4 and their location on the virion (Grubman *et al.* 2004).

The first structure of a picornaviral capsid was that of HRV14 (Rossmann *et al.* 1985). Several cryo-EM or X-ray crystal structures followed (e.g. for FMDV serotype O (Acharya *et al.* 1989) and PV1 (Hogle *et al.* 1985)). All structures revealed that all picornaviral capsids have an icosahedral symmetry with a triangulation number of $T=3$. The arrangement of VP1-VP3 gives rise to an uneven surface with a star-shaped plateau at the fivefold axis, surrounded by a deep depression (canyons) and another protrusion at the threefold axis (Fields *et al.* 2007). This canyon is normally referred to as the receptor binding site for entero- and cardioviruses but is not found on the surface of FMDV (Acharya *et al.* 1989). Another unique feature of FMDV is the presence of a pore at the five-fold axis, which allows small molecules to enter (Jackson *et al.* 2003). In contrary to some picornavirus species, the FMDV capsid dissociates at low pH and cannot pass the intestinal tract as the acid stable poliovirus or certain enteroviruses.

1.4 Life Cycle

1.4.1 Attachment and cell entry

Studies by Moore and Cowan (Moore *et al.* 1987) showed that trypsin treatment of FMDV results in noninfectious viral particles due to their inability to attach to cells. Further experiments revealed that trypsin cleaves VP1 at position Arg144 (Robertson *et al.* 1983), which suggests that the region around Arg144 might interact with the cell surface receptor. Subsequent studies on the binding of fibronectin to cells revealed the tri-peptide sequence Arg-Gly-Asp (RGD) as the cellular recognition site for the fibronectin-receptor (Pierschbacher *et al.* 1984). This sequence is also found in FMDV VP1 (Burman *et al.* 2006) and shown to be highly conserved amongst different FMDV

strains (Pfaff *et al.* 1988). The fibronectin-receptor belongs to the integrin family (Baxt *et al.* 1990) and consist of two subunits (α and β) that are bound at the cell surface. To confirm the interaction between the conserved sequence and the receptor, small peptides containing RGD were used to inhibit the binding of virus to the cells (Baxt *et al.* 1990, Fox *et al.* 1989). Site directed mutagenesis of the RDG sequence led to noninfectious viral particles, thus verifying former findings (Leippert *et al.* 1997, Mason *et al.* 1994, McKenna *et al.* 1995). $\alpha_v\alpha_3$ integrin was the first receptor identified to bind FMDV (Berinstein *et al.* 1995). However, it has been demonstrated that FMDV can also interact with $\alpha_v\alpha_6$ (Jackson *et al.* 2000) and $\alpha_v\alpha_1$ (Jackson *et al.* 2002). Certain FMDV strains also use heparan sulfate as cellular receptor (Jackson *et al.* 1996).

The exact process of entry and uncoating of FMDV is still vague but Carrillo *et al.* (Carrillo *et al.* 1985, Baxt *et al.* 1987) suggest a breakdown of the virion into subunits upon entry of acidic endosomes.

1.4.2 Translation and processing of the viral polyprotein

Replication of picornaviruses occurs entirely in the cell cytoplasm (figure 4). Following uncoating of the viral particle, the RNA is released into the cytoplasm. As picornaviruses are single-stranded positive sense RNA viruses, viral RNA serves as mRNA in the host cell. Picornaviral RNA cannot be copied by any cellular RNA polymerase and the viral capsid does not carry any viral enzymes. Therefore, picornaviral translation needs to occur before RNA replication (Fields *et al.* 2007). In contrary to host mRNA, viral mRNA does not contain a 7-methylguanosine cap at the 5' end (Martínez-Salas *et al.* 1999) and initiates protein synthesis internally at the IRES via a cap-independent mechanism (Belsham *et al.* 1990). As only one ORF is found on picornaviral genomes, translation results in the production of a single polyprotein. This polyprotein undergoes a series of cleavages producing structural and non-structural proteins (figure 1). Due to co-translational processing, no polyprotein is observed in infected cells (Fields *et al.* 2007).

The cleavage reactions are performed by two virally encoded proteases, Lb^{pro} and 3C^{pro}. The cascade of cleavages is initiated by Lb^{pro}, which cleaves between its own C-terminus and the N-terminus of VP4 (figure 2) (Guarné *et al.* 1998). The 2A protein of FMDV, an 18 amino-acid peptide, has no proteolytic activity. Nevertheless, the polyprotein is processed between the C-terminus of 2A and the N-terminus of 2B. A putative ribosomal skip was proposed as the mechanism that causes the interruption of the polyprotein chain (de Felipe *et al.* 2003). All remaining cleavages are performed by 3C^{pro} or its

precursor 3CD^{pro}. 3C^{pro} and its precursor cleave at dipeptide sequences as Gln-Gly, Glu-Gly, Gln-Leu and Glu-Ser (Birtley et al. 2005).

The last processing event is the maturation cleavage of VP0 into VP4 and VP2 in the already assembled viral particle. This process is thought to be autocatalytic requiring the presence of viral RNA and a conserved histidine in VP2 that initiates the nucleophilic attack (Basavappa et al. 1994, Curry et al. 1997, Hindiyyeh et al. 1999).

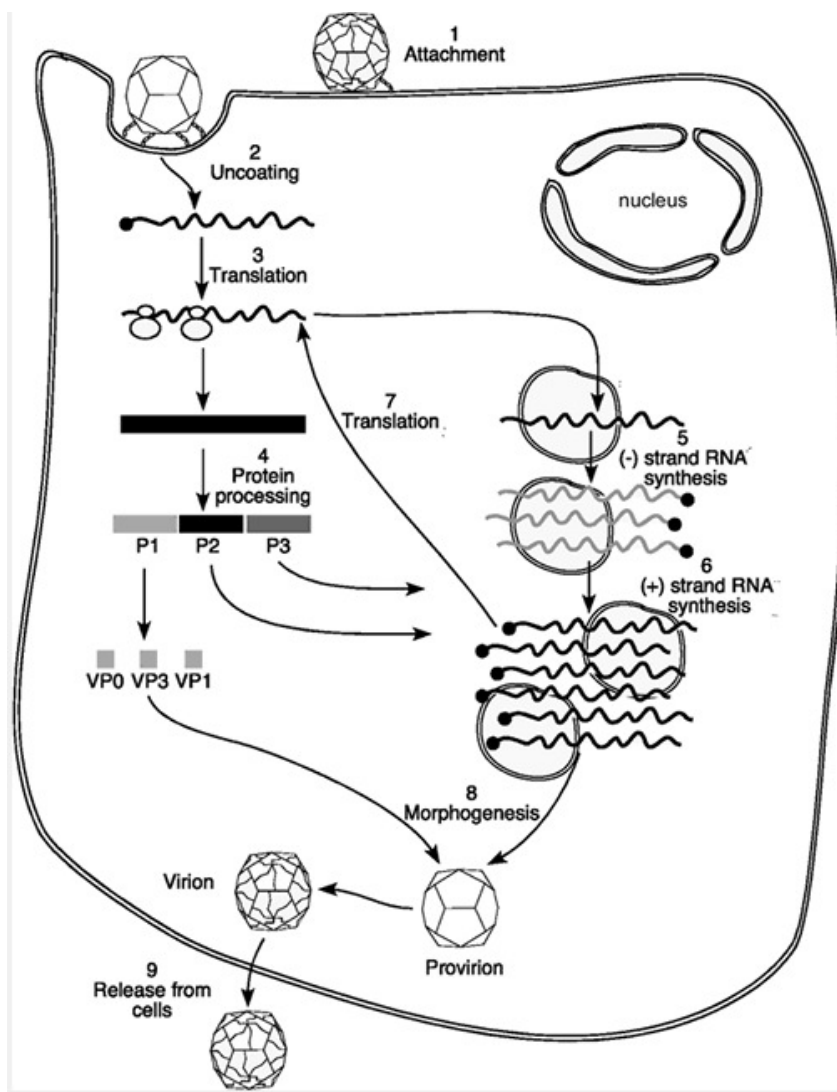


Figure 4: Schematic overview of the picornaviral life cycle. Virus attaches to a host receptor and enters the cell (1). After uncoating, the viral genomic RNA is released and VPg is removed from the RNA (2). RNA is first translated into a single polypeptide (3) that is co-translationally cleaved into viral proteins (4). RNA replication is carried out at membranous vesicles where the minus strand intermediate is synthesized by 3D^{pol} (5). This intermediate is used to produce new plus strand RNAs (6), which are packed into the viral particles (8) and released after maturation cleavage by lysis (9) (Fields et al. 2007).

1.4.3 Genome replication

Besides protein synthesis, the viral RNA is used as template for viral replication. Thus, due to this double function the viral genome needs to be recognized by the cellular translation machinery and the viral replication apparatus (Herold *et al.* 2001). Even though this process has been studied in other picornaviruses than FMDV, most of it is proposed to be similar (Paul *et al.* 2002). Picornaviral genome replication is a highly extensive process including all viral proteins except the structural ones. Evidence indicates that also host cell proteins are essential for picornaviral replication (Diez *et al.* 2000). A certain critical concentration of 3CD^{pro} is thought to initiate the shift between translation and transcription, as those two processes do not occur simultaneously (Gamarnik *et al.* 1998, Barton *et al.* 1999). Accumulated 3CD^{pro} then binds to the 5' cloverleaf structure and promotes transcription. Whether this mechanism proceeds similarly in FMDV is unknown. However, in FMDV infected cells 3CD^{pro} is rapidly cleaved into 3C^{pro} and 3D^{pol} (Fields *et al.* 2007) which would argue against it.

Picornaviral RNA replication occurs in the cytoplasm at virus induced vesicles derived from membranes of the endoplasmic reticulum and the Golgi apparatus (Monaghan *et al.* 2004). RNA synthesis of the negative strand is initiated at the 3' end of positive stranded RNA viruses. In picornaviruses, the 3' poly(A) tail is proposed to participate in RNA replication (Herold *et al.* 2001). Furthermore, Herold *et al.* demonstrated that ribonucleoprotein (RNP) complexes formed at the 3' and 5' ends of genomic RNA interact via a protein bridge. This interaction of the RNP complexes is thought to cause the circularization of the viral genome and thus be essential for the translation initiation of negative sense viral RNA (figure 5).

Following the initiation, the viral RNA dependent RNA polymerase 3D^{pol} synthesizes a minus strand RNA intermediate. As 3D^{pol} is a primer-dependent enzyme, it is not able to initiate RNA synthesis on a naked RNA strand. VPg is thought to be involved in the initiation of RNA replication by priming 3D^{pol} (Paul *et al.* 2003).

The generated minus strand forms fully double stranded RNA replication intermediates, the replicative form, which serve as template for the production of large excess of positive stranded RNA (Lescar *et al.* 2009).

Several secondary structure elements of the viral RNA are also involved in picornaviral replication. The cloverleaf at the 5' UTR (Andino *et al.* 1990) and a stem loop in the 3' UTR (Pilipenko *et al.* 1992a) assure correct recruitment of the viral RNA to the replicative complex.

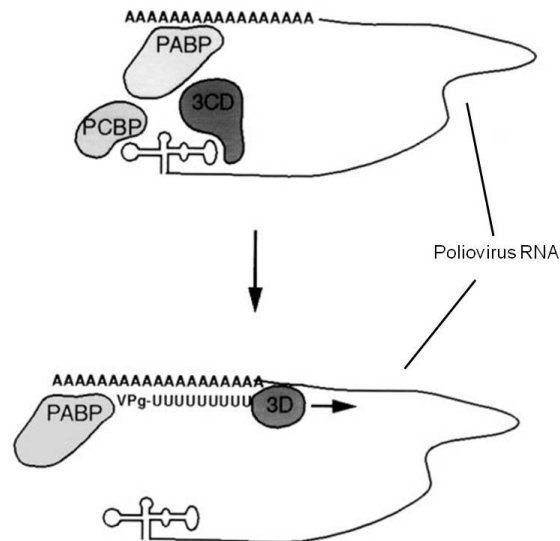


Figure 5: Model of translation initiation of negative strand RNA synthesis in poliovirus replication. After the translation, the viral polymerase precursor 3CD binds together with poly(A) binding protein (PABP) and the cellular factor poly(rC) binding protein (PCBP – a component of the RNP complex) to the 5' cloverleaf structure. These interactions bring the viral polymerase in close proximity to the 3' poly(A) tail thus initiating translation (Herold *et al.* 2001).

The cis replicative element (*cre*), a stem loop found in the 5' UTR of FMDV, contains a conserved AAACA motif within the loop. *Cre* functions as template for the uridylylation of VPg to generate VPgUpU_{OH} (Steil *et al.* 2009). This is feasible because of the presence of two adenosine residues in the loop of the *cre* that allow the viral RNA-dependent RNA polymerase 3D^{pol} to add two uridine residues to the tyrosine residue of VPg. VPg and VPgUpU_{OH} function as primer for the the RNA-dependent RNA polymerase 3D^{pol} (Steil *et al.* 2009). Although, the *cre* element has similar functions in different picornaviruses, it is located in distinct regions of the genome. The *cre* sequence of poliovirus was found in the protein-coding region of the 2C protein (Goodfellow *et al.* 2000) while in human rhinovirus 14 the *cre* sequence is encoded in the P1 region (McKnight *et al.* 1998).

1.4.4 Assembly and release of the virus

The final steps of the viral life cycle are the encapsidation of newly synthesized plus stranded RNA linked to VPg (Novak *et al.* 1991) and the maturation cleavage of VP0 into VP2 and VP4. The building blocks of the capsid are protomers, which contain one copy of VP0, VP1 and VP3. Five protomers (5S) can assemble to form a pentamer (14S), of which 12 are used to form the final capsid structure. Currently, there are two modes of assembly. One suggests the formation of empty capsids (80S) into which RNA is inserted subsequently (Jacobson *et al.* 1968). The second proposes that pentamers directly interact with the RNA, forming the provirion (150S) (Nugent *et al.* 1995; figure 6).

In presence of viral RNA, VP0 is cleaved into VP2 and VP4 resulting in the mature virion (160S) (Basavappa *et al.* 1994).

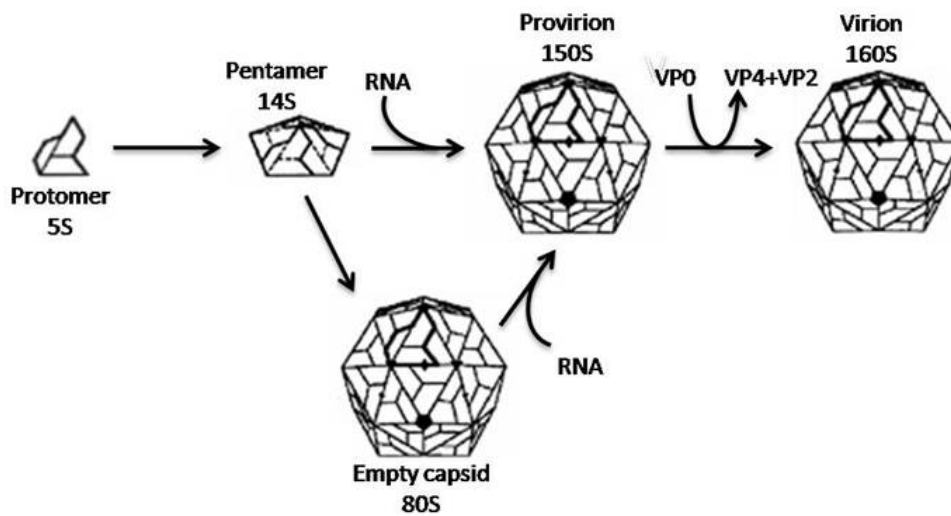


Figure 6: Assembly of the viral particle. Five protomers are combined to form the 5S subunit that further builds the 14S pentamer. Two mechanisms are possible: the internalization of the RNA, after formation of the 80S capsid or the direct interaction of the 14S pentamers with the RNA giving rise to the provirus. The maturation cleavage of VP0 into VP4 and VP2 yields mature 160S virions. (Rotbart *et al.* 1995)

1.5 Leader protease (Lb^{pro})

The proteolytic activity of the leader protein is a specific feature of aphtho- and erboviruses. It is encoded at the very 5' end of the genome and represents the second protease besides 3C^{pro}. Initiation of protein synthesis can start at two different AUG start codons lying 84 nucleotides apart (Carrillo *et al.* 2005). Translation gives rise to two species of the leader protein, Lab^{pro} and Lb^{pro}. Both variants display the same enzymatic properties, even though Lb^{pro} is 28 amino acids (aa) shorter than Lab^{pro}. Throughout this work Lb^{pro} of FMDV O1_k was used, as it was shown that it is the physiologically active protease (Cao *et al.* 1995).

1.5.1 Structure of Lb^{pro}

Lb^{pro} was found to be a cysteine protease possessing a papain-like fold (Guarné *et al.* 1998). Even though papain and Lb^{pro} share only 15% of sequence identity, the residues of the active site are conserved. Figure 7 shows the X-ray crystal structures of Lb^{pro} and papain. Both consist of a compact globular domain organized in two subdomains with the active site residues Cys51 and His148 of Lb^{pro} and Cys25 and His159 of papain at the interface of the N-terminal and C-terminal domain. The N-terminal domain of Lb^{pro} contains four α -helices (α_1 - α_4) and two short antiparallel β -strands (β_1 and β_2). The nucleophilic Cys51 is located at the N-terminal end of α_1 which runs perpendicularly to α_3 . The C-terminal subdomain represents a fold composed of only mixed β -sheets, one parallel (β_3 - β_4) and six antiparallel (β_4 - β_9) β -strands. His148 is located at the connecting turn of β_5 and β_6 . In Lb^{pro}, the catalytic triad is completed by the conserved Asp163, which keeps Cys51 and His148 in the correct orientation to each other. Asparagine completes the catalytic triad in papain (Guarné *et al.* 2000).

Despite the similarity to papain, Lb^{pro} contains 18 aa protruding from the globular domain, with no equivalent in papain. This long C-terminal extension (CTE) is a highly flexible region involved in self-processing and the cleavage of eukaryotic initiation factor 4G (eIF4G) (Glaser *et al.* 2001, Cencic *et al.* 2007). Cencic *et al.* showed that a version of Lb^{pro} with a shortened CTE was not able to self-process. Another unusual property of the CTE is its insertion into the active site of the neighboring molecule giving rise to a stable homodimer (Guarné *et al.* 1998). Residues Lys201 and Leu200 of the CTE, at position P₁ and P₂, provide strong interactions with the substrate-binding site (The nomenclature of the binding and corresponding cleavage sites on the enzyme is that from Berger and Schechter 1970).

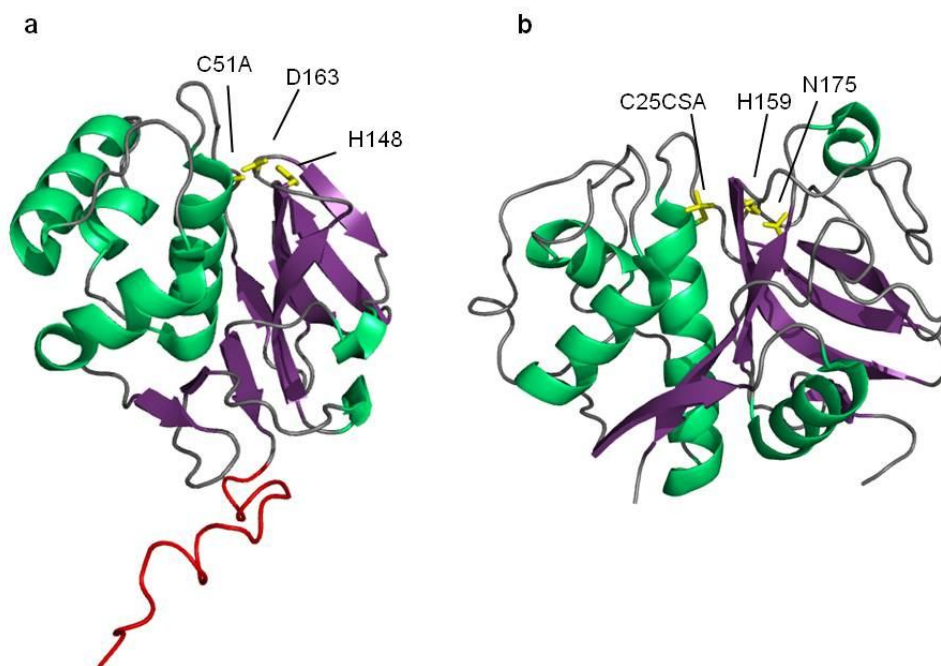


Figure 7: Crystal structures of (a) Lb^{pro} (Guarné *et al.* 1998) and (b) papain (Kamphuis *et al.* 1984). α - helices and β -sheets are shown in green and purple, respectively. The residues of the active site are represented as yellow sticks. For crystallization, Cys51 of Lb^{pro} was mutated to Ala (C51A). In papain, the active Cys25 was mutated to cysteinesulfonic acid (C25CSA). The 18 aa CTE of Lb^{pro} is depicted in red. Created with PyMOL (Delano, 2002) using the PDB ID codes 1QOL (a) and 9PAP (b).

Residues P₁ to P₃ are bound to the active site in an extended conformation whilst P₄ to P₇ are bound in a tight turn comparable to a 3₁₀ α -helix (Santos *et al.* 2009).

The S₁ subsite forms a narrow cleft which prefers a basic residue at the P₁ position. In Lb^{pro} Lys201 (P₁) points away from the active site and is embedded between residues Glu96 and Glu147. The side chains of other residues tested at this position clashed with the aliphatic side chains of these glutamates and were thus not accepted.

As in other cysteine proteases (e.g. papain), the specificity of Lb^{pro} is mainly determined at the S₂ position. Therefore, only leucine, valine and, to a certain extent, isoleucine, are accepted (Santos *et al.* 2009). Figure 8 shows that Leu200 (P₂) is completely buried in the deep cavity formed by several hydrophobic residues (side chain residues P99, P100, A101, I141, L143, A149 and L178 and the main-chain atoms of W52, F142, and H148). Kuehnelt *et al.* suggested that Lb^{pro} is able to discriminate between Leu and Phe at the P₂ position and can also exclude acidic residues at P₃. Especially the P₂ Leu200 to Phe substitution affected self-processing (Kuehnelt *et al.* 2004). Subsequently, Mayer and colleagues (Mayer *et al.* 2008) examined the S₂ pocket for residues which might clash with Phe at P₂. Molecular modeling experiments showed that the side chain of Leu143 might prevent the binding of Phe. Therefore, leucine was replaced by the smaller side chain of alanine to test whether this would improve the

acceptance of Phe. An additional Leu143 to Ala substitution in a Leu200Phe variant restores the self-processing event. As alanine provides more space in the S₂ pocket, sterical hindrances caused by Leu143 could be the reason for the exclusion of Phe. Sequence alignments revealed that Leu143 is not invariant, but the only other residue occurring at this position is methionine, another hydrophobic residue with a bulky side chain (Mayer *et al.* 2008).

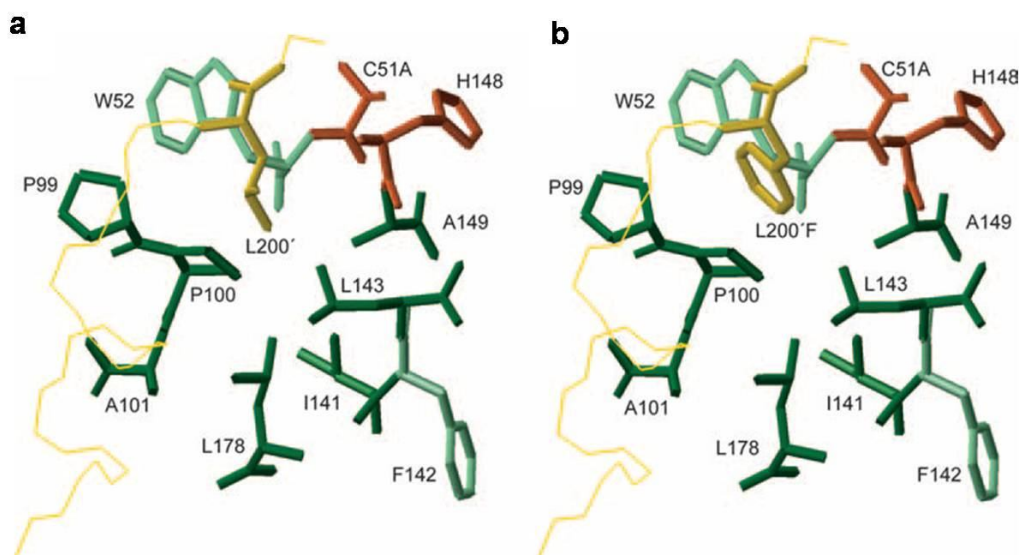


Figure 8: The structure of the S₂ pocket of Lb^{pro}. (a) Structure of the wild-type S₂ pocket showing Leu200. (b) Leu200 has been substituted to Phe by molecular modeling. The residues of the hydrophobic pocket are presented in dark green. The backbone of the CTE and Leu200 shown in yellow. Active site residues are shown in red (Cys51 is replaced by Ala in the crystal structure). Main chain interactions contributing to the formation of the S₂ pocket are in light-green (Mayer *et al.* 2008).

Hence, Leu143 seems to be important for the maintenance of the Lb^{pro} specificity as especially substrates with phenylalanine at the P₂ residues are not processed. The number of possible substrates is therefore restricted, which is supported by the fact that besides the two homologues of eIF4G and the viral polyprotein (Santos *et al.* 2009), no other substrate has been identified. Furthermore, the observed dimer formation might inhibit substrate cleavage and reduce the number of substrates even further.

Lys199 (P₃), pointing away from the substrate binding site, and Arg198 (P₄) occupy the pockets of the S₃ and S₄, respectively. It was demonstrated that Asn and Lys are preferred at P₃, whereas Asp and Arg are less favored. In contrast, Arg is accepted in the S₄ pocket forming hydrogen bonds with Gln146 and Van der Waals interactions with Leu143 (Santos *et al.* 2009).

Although, there is no real binding site for the P₅ residue, Gln197 is possibly involved in the formation of the 3₁₀-turn mentioned above.

The site for P₆ recognition is composed of several hydrophobic residues able to accept the wild-type Val196 as well as Phe, Pro and Ser. Lys195 at the P₇ position forms Van der Waals interactions with Leu143.

Additionally, some residues contribute to more than one specific pocket. Leu178 is involved in the formation of to the S₂ and the S₆ pocket, whereas Leu143 is essential for the formation of the S₂ and S₄ subsite and forms Van der Waals interactions with Lys195 (Santos *et al.* 2009).

1.5.2 Functions and enzymatic activities of Lb^{pro}

As already described above, Lb^{pro} is a highly specific protease, cleaving only three known substrates. These are the viral polyprotein at the Lb^{pro}-VP4 junction and the two homologues of eIF4G. Table 1 summarizes the cleavage sites recognized by Lb^{pro}. A consensus sequence cannot be determined, as the cleavage sites differ strongly from each other. However, the related sequence AFADFGX^RQTPGG in eIF4GII is not cleaved by Lb^{pro}. The presence of the aspartic acid at the P₃ position or phenylalanine at P₂ prevent the cleavage.

Substrate	P-region	P'-region
Lb ^{pro} -VP4 ¹⁾	VQRKLK ²⁰¹ ↓	²⁰² GAGQSS
eIF4GI ²⁾	SFANLG ⁶⁷⁴ ↓	⁶⁷⁵ RTTLST
eIF4GII ³⁾	PLLNVG ⁷⁰² ↓	⁷⁰³ SRRSQP
eIF4GII not cleaved ⁴⁾	AFADFG ⁶⁸⁶ X	⁶⁸⁷ RQTPGG

Tabel 1: Overview of cleavage sites recognized by Lb^{pro}. Sequences are taken from ¹⁾Strebel *et al.* 1986, ²⁾Foeger *et al.* 2002, ³⁾Gradi *et al.* 2004 and ⁴⁾Kuehnel *et al.* 2004, respectively.

1.5.2.1 Self-processing

The initial cleavage event on the FMDV polyprotein occurs co-translational and is achieved by the Lb^{pro}, which frees itself by cleaving between its own C-terminus and the N-terminus of VP4 at the sequence VQRKLK²⁰¹↓²⁰²GAGQSS (Strebel *et al.* 1986). This reaction could be either intra- or intermolecular (cis or trans, respectively) (Glaser *et al.* 2001). X-ray crystal and NMR structures show the CTE bound to the active site of the neighboring molecule, arguing for an intermolecular self-processing event whereas several characteristics suggest an intramolecular reaction as molecular modeling studies consider the possibility of the CTE to fold back and bind to its own active site (Cencic *et al.* 2007). The high mobility of the CTE would argue for this possibility. Furthermore,

Glaser and colleagues already demonstrated that intramolecular self-processing and intermolecular cleavage of eIF4GI is favored over intermolecular self-processing and intramolecular cleavage of eIF4GI (Glaser *et al.* 2001).

1.5.2.2 eIF4G binding and cleavage

The only host cell proteins cleaved by Lb^{pro} during FMDV infection are the homologues of eIF4G. This cleavage disrupts the scaffolding function of eIF4G (see 1.6.1), leading to the inhibition of host cell protein synthesis, also termed host cell shut off. Viral translation, however, remains unaffected as it initiates via an IRES.

Most of the work published on eIF4G processing was on eIF4GI. As the crucial characteristics are conserved, the results are transferable to a large extent.

The cleavage of eIF4GI and eIF4GII is very effective *in vivo* and *in vitro*, but the concentration of Lb^{pro} in infected cells at the time of eIF4G cleavage is much lower than the protease needed for *in vitro* experiments (Glaser *et al.* 2000, Kirchweiger *et al.* 1994). These findings led to the suggestions that Lb^{pro} activates latent cellular proteases (Wyckoff *et al.* 1990, Wyckoff *et al.* 1992). However, no such cellular protease could be identified. Furthermore, Ohlmann *et al.* suggest that the eIF4G-eIF4E complex is the actual substrate for Lb^{pro} (Ohlmann *et al.* 1997). While 2A^{pro} of HRV2 only interacts with eIF4GI when eIF4E is present, Lb^{pro} is able to cleave eIF4G on its own. Nevertheless, binding of Lb^{pro} to eIF4G is enhanced upon complex formation with eIF4E due to an unfolded-to-folded transition of eIF4G as observed in yeast eIF4GI (Hershey *et al.* 1999, Gross *et al.* 2003, Foeger *et al.* 2002). These rearrangements are thought to cause the exposure of the Lb^{pro} cleavage sequence on eIF4G thus facilitating the accession of Lb^{pro}. Therefore, the binding of eIF4E to eIF4G is considered to enhance the cleavage process.

The interaction site of eIF4GI with the Lb^{pro} is located at a distinct site away from the cleavage site (Table 1). Amino acids 645-657 on eIF4GI were demonstrated to act as the binding site for Lb^{pro} (Foeger *et al.* 2005). Three basic residues (Lys643, Lys646 and Arg650) are conserved between human eIF4G homologues and several mammalian species. Foeger *et al.* demonstrated that substitution of any of these residues with Ala influences the interaction with Lb^{pro} differently (Foeger *et al.* 2005). The replacement of Arg650 and Lys646 reduces the binding efficiency by 75% and 50-75%, respectively. In contrast, the substitution of Lys643 lowers the binding only by 10-20%. These results would imply that the three basic residues might be recognized by residues of the CTE thus facilitating interaction.

The eIF4G binding domain on Lb^{pro} is found 25 Å distant from the active site and involves Cys133 and the residues 183-195 of the CTE. The two acidic residues Asp184 and Glu186 of the CTE are conserved in all seven FMDV serotypes and play an important role in the binding of eIF4GI. Investigation of mutants Asp184Ala and Glu186Lys showed a delay of eIF4GI cleavage. Additionally, the double mutant Gln185ArgGlu186Lys delays eIF4GI cleavage even further. This suggests that the overall charge of this region might be important for eIF4GI interaction. Interestingly, neither of these mutations inhibits eIF4GI cleavage completely or affects Lb^{pro} self-processing. In contrast, Cys133 is not only involved in binding of eIF4GI but also affects cleavage as shown by site-directed mutagenesis (Foeger *et al.* 2002).

Figure 9 represents the arrangement of Lb^{pro} residues involved in eIF4GI recognition. Residues Asp184 and Glu186 are distal to the Lb^{pro} active site and 12 Å from Cys133. Therefore, interactions with any residues of the globular domain or the CTE seem unlikely, as both residues point away from the molecule. Hence, the inhibition of eIF4GI cleavage is possible without affecting self-processing whereupon self-processing is no prerequisite for eIF4G cleavage (Glaser *et al.* 2001).

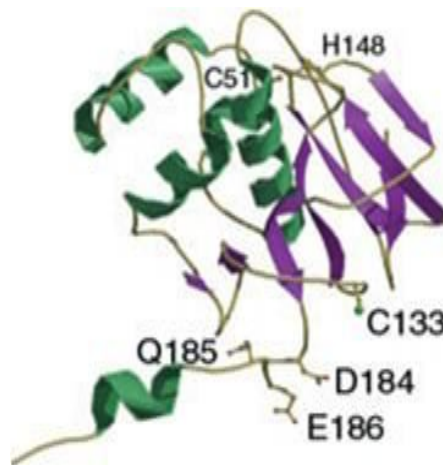


Figure 9: Lb^{pro} residues involved in eIF4GI recognition. α -helices and β -sheets are shown in green and purple respectively. The catalytic residues C51 and H148 of Lb^{pro} and C133, Q185, D184 and E186 are shown as sticks (Foeger *et al.* 2005).

1.5.2.3 Interference of Lb^{pro} with innate immunity

Viruses evolved different mechanisms to overcome the early host cell immune response. FMDV wild-type was shown to, on the one hand, block host protein synthesis due to the inhibition of cap-dependent translation (Chinsangaram *et al.* 2001). On the other hand, the virus inhibits the induction of IFN- α/β mRNA (de los Santos *et al.* 2006). De los Santos and colleagues recently demonstrated that Lb^{pro} is directly associated with

the regulation of IFN- β expression. Thereby, Lb^{pro} degrades p65/RelA, a subunit of the nuclear factor κ B (NF- κ B) (de los Santos *et al.* 2007). The direct involvement of Lb^{pro} was demonstrated by experiments with a FMDV without the leader protease sequence which did not cause any degradation of P65/RelA. Thus, the activity of NF- κ B is directly affected which in turn is required for the expression of IFN- β . Furthermore, these experiments may indicate that P65/RelA is cleaved by Lb^{pro} (de los Santos *et al.* 2007). A putative Lb^{pro} cleavage site was found by analyzing the amino acid sequence of p65/RelA. However, no degradation products could be detected which could indicate that p65/RelA cleavage does not occur during FMDV infection.

Nevertheless, the leader protease of FMDV is crucial for the survival of the virus in the host cell and has evolved mechanisms to antagonize the innate immune response at multiple levels.

1.6 Translation initiation

1.6.1 Cap-dependent translation

Eukaryotic cells synthesize their proteins using a cap-dependent translation process. The 7-methyl-guanosine cap at the 5' end of cellular mRNAs is on the one hand essential for stabilization and RNA splicing. On the other hand, the cap structure recruits eIF4E. eIF4E then interacts with eIF4G, which recruits the 40S ribosomal subunit via binding the eukaryotic initiation factor 3 (eIF3) (figure 10).

Translation is a stepwise process and starts by formation of the 43S preinitiation complex comprising a 40S ribosomal subunit, eukaryotic initiation factor (eIF) 1 (eIF1), eIF1A, eIF3, eIF2-GTP-Met-tRNA_i^{Met} and probably eIF5 (Hershey *et al.* 2000, Lopez-Lastra *et al.* 2005). The mRNA becomes activated by attachment of the eIF4F complex, consisting of eIF4E, eIF4G and eIF4A. The 40S ribosomal subunit of the preinitiation complex then binds to the already activated mRNA and starts to unwind and scan the mRNA for the initiation codon (Hentze *et al.* 1997, Pain *et al.* 1996). The joining of the 60S ribosomal subunit completes the 80S initiation complex and translation can occur.

The eukaryotic initiation factors 4E and 4G of the eIF4F complex are especially relevant to this work.

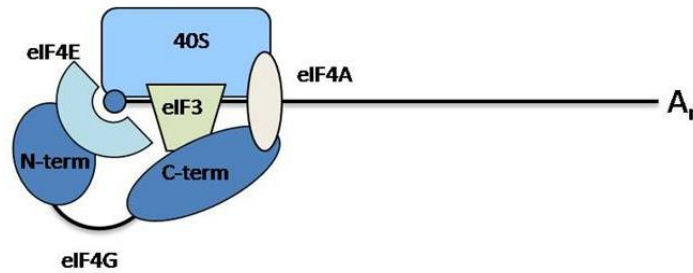


Figure 10: Translation initiation factors involved in cap-dependent translation of picornaviruses. During host cell translation initiation, eIF4G acts as a scaffold bringing the different parts of the initiation complex together and recruiting the 40S subunit to the mRNA. eIF4E binds the cap structure of the 5' end of the mRNA and recruits the eIF4G (Fields *et al.* 2007).

1.6.1.1 Eukaryotic initiation factor 4G (eIF4G)

eIF4G is a 220 kDa protein, formerly named p220 or eIF-4γ. Human eIF4GI was first isolated as part of a large protein complex which was able to restore protein synthesis in lysates derived from poliovirus-infected HeLa cells (Tahara *et al.* 1981). This complex was identified as translation initiation complex eIF4F, in which eIF4G acts as scaffold holding different components of the complex together (LeFebvre *et al.* 2006). Two homologues of human eIF4G, termed eIF4GI and eIF4GII, are known which have 46% identity at the amino acid level and an overall similarity of 56% (Gradi *et al.* 1998). The homology is particularly high in the area of the central part where a stretch of 70 aa is conserved. Even though eIF4GII has a predicted molecular mass of about 176 kDa, both homologues migrate as a series of bands at 195 kDa when separated by SDS-PAGE (Lamphear *et al.* 1995). The later identified eIF4GII is a functional homolog that interacts with eIF4E, eIF4A and eIF3 as does eIF4GI. Moreover, eIF4GII was found to be present in the eIF4F complex and therefore is vital for translation initiation.

eIF4G consists of two domains, an N-terminal domain and a C-terminal domain. The N-terminal domain contains a binding site for the cap binding protein eIF4E (Mader *et al.* 1995) and the poly-A binding protein (PABP). Binding of the PABP leads to the circularization of the translation initiation complex (Imatka *et al.* 1998). In contrast, the C-terminal domain interacts with eIF4A, eukaryotic initiation factor 3 (eIF3) and protein kinase Mnk1 (Pyronnet *et al.* 1999). Hence eIF4G is vital for translation initiation as it coordinates the components required to build the preinitiation complex onto the messenger RNA. A schematic representation of eIF4GII is shown in figure 11.

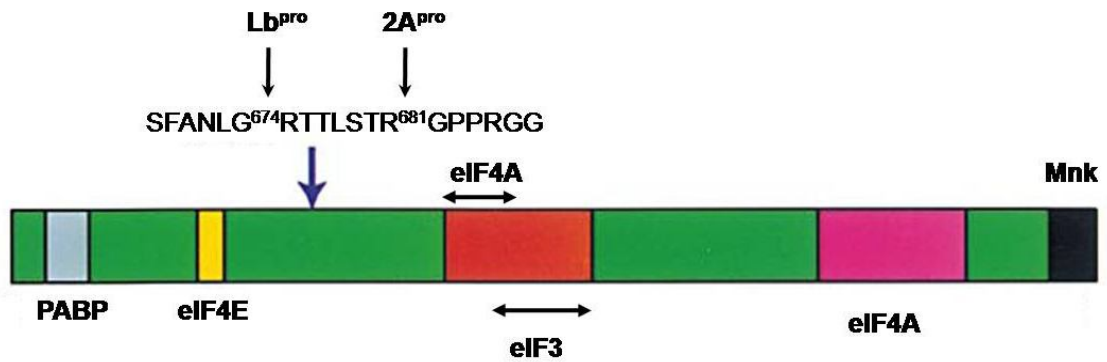


Figure 11: Domains of eIF4GII. Binding sites for different proteins are colored. The blue arrow indicates the cleavage sites for the picornaviral proteases 2A^{pro} and Lb^{pro} (adapted from Marcotrigiano *et al.* 2001).

Amino acids 605-718 were shown to be necessary for interaction of eIF4GII with eIF4E. This region contains the 13 conserved amino acids KQYDREFLLDFQF (622-634) binding eIF4E. Mader *et al.* tested different fragments of eIF4GII and their interaction with mouse eIF4E (Mader *et al.* 1995). A 49 aa region in eIF4G (aa 409-457) was demonstrated to be necessary and sufficient for binding to eIF4E. The binding motif was determined further to 12 aa that are highly conserved amongst yeast eIF4G homologues. Studies by Marcotrigiano showed that eIF4G binds the dorsal, convex surface of eIF4E, which is found distal to the cap binding pocket (Marcotrigiano *et al.* 1999) (figure 12).

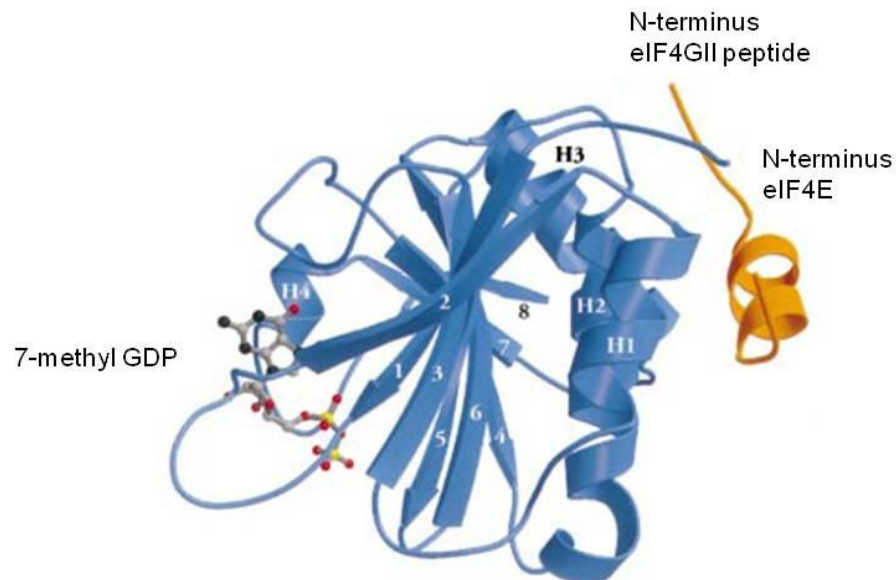


Figure 12: X-ray crystal structure of murine eIF4E bound to 7-methyl GDP and an eIF4GII peptide resulting in a ternary complex. Ribbon diagrams of eIF4E (blue) and the eIF4GII peptide (orange). The eIF4GII peptide interacts with the helices H1 and H2 of eIF4E. 7-methyl GDP as ball-stick-representation. The binding site of 7-methyl GDP lies 35 Å from the eIF4GII binding site. PDB ID code 1EJH (Marcotrigiano *et al.* 1999).

Several other proteins are known to bind this site as do the 4E binding proteins (4E-BPs) (figure 13). During the interaction of eIF4E with 4E-BPs, the recognition site for eIF4G is sterically blocked and the assembly of the translation machinery inhibited (Ptushkina *et al.* 1999).

Numerous proteins binding to eIF4E were found to have the conserved eIF4E binding motif YXXXXLΦ (X standing for any residue and Φ for any hydrophobic residue) (Volpon *et al.* 2006). This motif becomes structured and adopts an L-shaped conformation (extended chain/α helix) upon interaction with eIF4E (Marcotrigiano *et al.* 1999). Studies of 4E-BP1 revealed that it indeed is intrinsically unstructured without eIF4E. Further observations demonstrate the competition of eIF4G and 4E-BPs for the same surface on eIF4E.

As already shown in figure 11, eIF4GII is cleaved by the picornaviral proteases Lb^{pro} and 2A^{pro} during infection and causes inhibition of host cell protein synthesis (see 1.5.2.2).

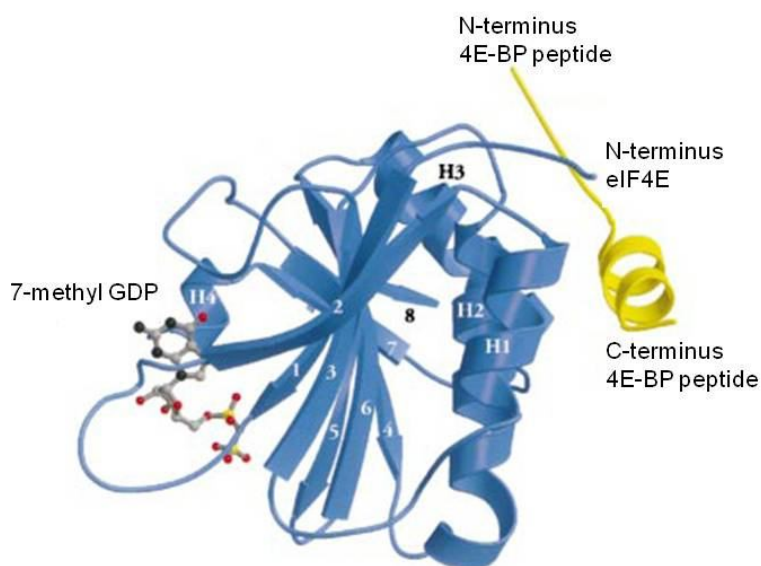


Figure 13: X-ray crystal structure of murine eIF4E bound to 7-methyl GDP and an 4E-BP peptide resulting in a ternary complex. Ribbon diagrams of eIF4E (blue) and the 4E-BP peptide (yellow). The 4E-BP peptide interacts with similar sites of eIF4E as the eIF4GII peptide. 7-methyl GDP as ball-stick-representation. The binding site of 7-methyl GDP lies 35 Å from the eIF4GII binding site. PDB ID code 1EJH (Marcotrigiano *et al.* 1999).

The only structural data available of eIF4GII is the X-ray structure of the conserved middle part of eIF4GII, aa 754-1003 (Marcotrigiano *et al.* 2001). This domain is known to interact with eIF4A and picornaviral IRESs. The structure was determined at 2.4 Å resolution, displaying a crescent-shaped protein composed of ten α-helices. The

helices are arranged as five antiparallel α helical pairs, called HEAT repeats (four proteins give rise to its name: **H**untingtin, **e**longation factor 3, protein phosphatase **2A** and yeast PI3 kinase **TOR1**). The repeats generate a double layer of α helices stabilized by salt bridges and Van der Waals interactions building a hydrophobic core (figure 14).

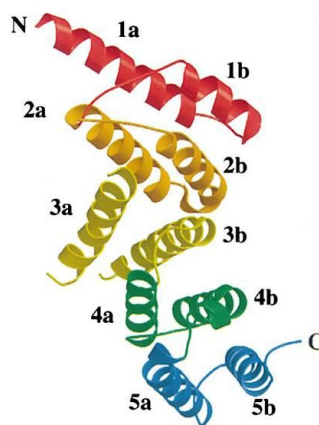


Figure 14: Ribbon diagram of the conserved central region (aa 745-1003) of human eIF4GII. View along the cylindrical axes of the α helices. The concave surface is built by the a helices on the right and the convex surface by the b helices to the left. PDB ID code 1HU3 (Marcotrigiano *et al.* 2001).

1.6.1.2 Eukaryotic initiation factor 4E (eIF4E)

Eukaryotic initiation factor 4E, as eIF4G, belongs to the eIF4F complex of translation initiation factors. Its function is to regulate mRNA translation and nuclear mRNA export by binding the 7-methyl-guanosine cap at the 5' end of cellular mRNA (Haghighat *et al.* 1997). Besides these processes, eIF4E is involved in oncogenic transformation (Sonenberg *et al.* 1998). eIF4E is regulated by phosphorylation, which was shown to reduce its cap-binding ability (Scheper *et al.* 2002). Unphosphorylated eIF4E, on the other hand, stimulates translation *in vitro* (Svitkin *et al.* 1996). The theoretical molecular weight of eIF4E is about 25 kDa. However, when analyzed by SDS-PAGE it runs at around 37 kDa (accession number: P06730).

eIF4E has been successfully cocrystallized with an eIF4GI peptide, different types of possible cap-structures (N7-alkylated cap, 7-methylGpppG, 7-methyl GTP, 7-methyl GDP), and 4E-binding protein 1 (4E-BP1) (Brown *et al.* 2007, Niedzwiecka *et al.* 2002, Tomoo *et al.* 2002, Marcotrigiano *et al.* 1997, Marcotrigiano *et al.* 1999). Structures of ternary complexes of eIF4E with either m7GpppA/4E-BP1 or with eIF4G peptide/7-methyl-GDP are available as well. Furthermore, Volpon and colleagues recently determined the cap-free structure of human eIF4E by NMR techniques (Volpon *et al.* 2006) (PDB ID 2GPQ).

Several structures of eIF4E bound to a cap-structure helped to identify the molecular contacts between them (Marcotrigiano *et al.* 1997, Marcotrigiano *et al.* 1999, Matsuo *et al.* 1997, Tomoo *et al.* 2003,). Murine eIF4E in the cap bound form resembles a cupped hand and consists of an eight-stranded antiparallel β -sheet supported by three long α -helices (Niedzwiecka *et al.* 2002). The cap-binding site of eIF4E is formed by a cavity on the molecules concave surface. During the binding of the cap analog m⁷GTP, the guanosine part is sandwiched between two conserved tryptophans (Trp56 and Trp102) holding the guanosine in place throughout the interaction. Furthermore, a third conserved tryptophan (Trp166) forms hydrogen bonds and Van der Waals interactions with the N7-methyl group (Marcotrigiano *et al.* 1997). The additional delocalization of the positive charge of the methyl group further stabilizes the stacking interaction by interaction with Glu103 of eIF4E. Furthermore, Volpon *et al.* demonstrated that cap addition modulates the conformation of the dorsal surface of eIF4E (Volpon *et al.* 2006).

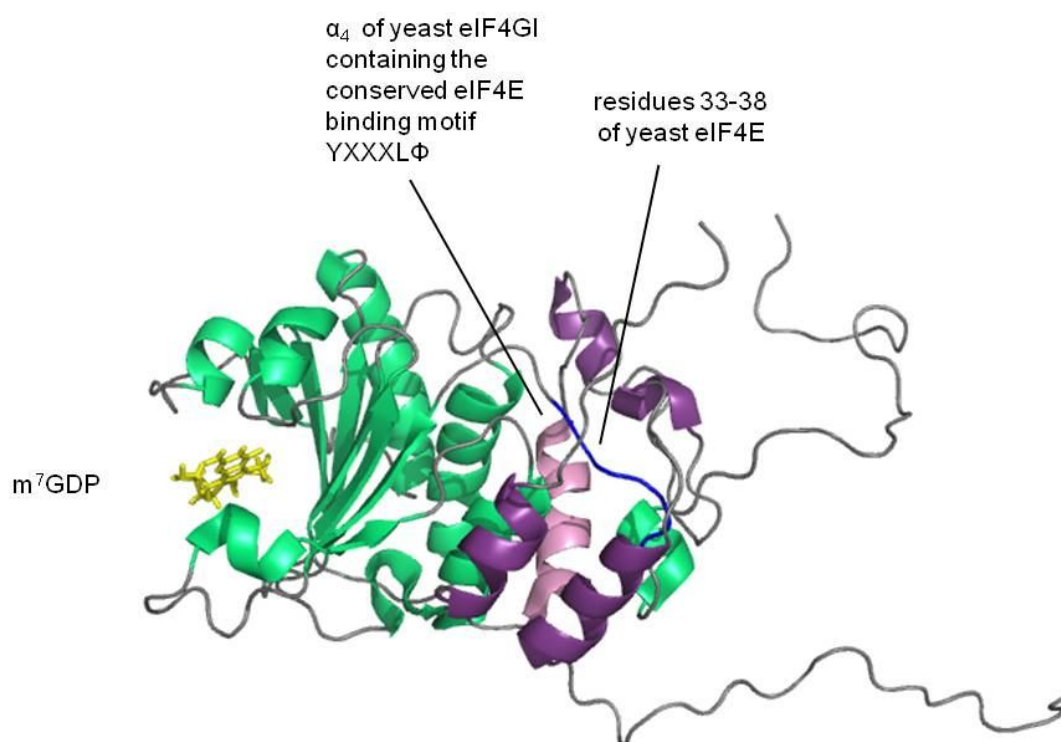


Figure 15: NMR structure of yeast translation initiation factor eIF4E in complex with m⁷GDP and eIF4GI (residues 393-490). Yeast eIF4E is shown in green and eIF4GI in purple. The m⁷GDP is represented as yellow sticks. The light pink helix displays α_4 of eIF4GI containing the conserved eIF4E binding motif YXXXL Φ . Residues 33-38 of eIF4E enclosed by eIF4GI are presented in blue (Gross *et al.* 2003). Created with PyMOL (Delano, 2002) using the PDB ID code 1RF8.

Gross *et al.* solved the structure of the ternary complex between yeast eIF4E, eIF4G (residues 393-490) and m⁷GDP using multidimensional NMR (Gross *et al.* 2003) shown in figure 15. eIF4G was shown to adopt a ring-shaped structure of five helical regions (α_1 - α_5) upon binding eIF4E. The consensus sequence YXXXL Φ is located in α_4 , which forms together with α_1 , α_2 , and α_5 a hydrophobic cavity enclosing the N-terminal residues 33-38 of eIF4E. Furthermore, this sequence was demonstrated to be sufficient for binding of eIF4E to eIF4G (Mader *et al.* 1995). Residues 23-32 of eIF4E emerge from the cavity and fold into a turn/helix/extended chain structure. The contacts of yeast eIF4E/eIF4G are found at the convex surface of eIF4E as observed for the murine eIF4E/eIF4GII peptide complex (Marcotrigiano *et al.* 1999). Several residues of all five helices of eIF4G interact with eIF4E. These residues are highly conserved or similar in mammals. Additionally, hydrophobic and charged interactions between α_5 of eIF4G and β_2 and α_3 of eIF4E are present.

1.6.2 Cap-independent translation and host-cell shutoff

Viruses are dependent on host cells for their replication and have evolved specific strategies to survive. Picornaviruses, except coronaviruses, developed a mechanism called host cell shutoff, which inhibits host cell protein synthesis and allows the virus to take over the host cell (Bedard *et al.* 2004).

In contrast to eukaryotic mRNAs, viral mRNAs lack a 5' cap structure recognized by the cap binding protein eIF4E. The recognition of the cap structure is important for the process of protein synthesis initiation. Upon viral infection, the cap-dependent translation (see 1.6.1) is prevented due to the cleavage of eIF4G by picornaviral proteases Lb^{pro} and 2A^{pro}. The disruption of the eIF4G scaffolding function separates the eIF4E binding domain from the C-terminal domain which interacts with eIF3 that recruits the 40S ribosomal subunit. The cleavage of eIF4G by picornaviral proteases to block host cell translation was first observed in PV infected HeLa cells (Etchison *et al.* 1982).

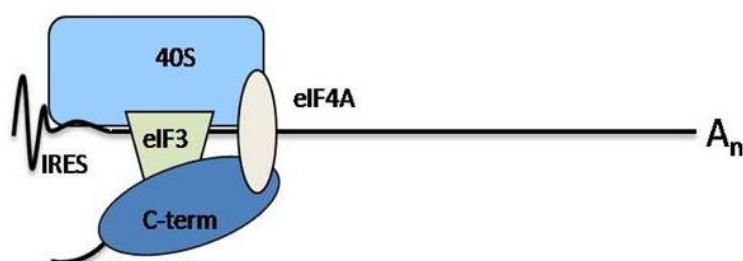


Figure 16: Translation initiation factors involved in cap-independent translation of picornaviruses. eIF4G is cleaved during viral infection by Lb^{pro} or 2A^{pro}. Hence, the complex can no longer recruit capped mRNA. However, the cleaved C-terminal part of eIF4G is sufficient to initiate viral translation via an IRES (Fields *et al.* 2007).

2 Aim of the work

The reason for exploring Lb^{pro} is to further understand the specificity and the reactions to design a possible inhibitor. Kleina *et al.* 1992 demonstrated that the inhibition of Lb^{pro} activity in cells causes a 1000-fold reduction in virus yield. Thus, if the self-processing reaction is prevented, Lb^{pro} is thought to remain connected to VP4. Accordingly, the assembly of viable viral particles might not be feasible anymore. Previous experiments showed that this is true for 2A^{pro} of poliovirus (Crowder *et al.* 2005). Therefore, the design of a functional inhibitor of Lb^{pro} requires not only the X-ray crystal structure, but also knowledge about its interaction with the substrates.

The aim of this work was to closer investigate the specificity of the Leu143 residue of FMDV Lb^{pro}. This residue was formerly shown to be involved in the formation of the hydrophobic S₂ pocket by binding Leu200 (Kuehnle *et al.* 2004). Furthermore, Lb^{pro} as a cysteine protease gains its specificity mainly at the S₂ position, only accepting leucine, valine and, to a certain extent isoleucine, (Santos *et al.* 2009). This specificity was attributed to Leu143 as it is pivotal for the discrimination of all other residues especially phenylalanine. The double mutant Leu200PheLeu143Ala showed that in this case Phe is accepted and even processed to wild-type levels. Therefore, Leu143 is essential for the conservation of the Lb^{pro} specificity and one reason for the restricted number of substrates.

How does an active Lb^{pro} L143A interact with its substrate eIF4GII? Based on the fact that only the two homologues of eIF4G are cleaved by wild-type Lb^{pro}, we were interested in the change of specificity using Lb^{pro} L143A. We assumed that the related cleavage sequence present in eIF4GII and not recognized by Lb^{pro} wt, might be cleaved by the mutant Lb^{pro}. This sequence contains Phe at P₂ and Asp at P₃, which should be accepted by Lb^{pro} L143A. This would further shed light on the interaction of the leader protease with its substrate.

What are the cleavage differences between Lb^{pro} wt and Lb^{pro} L143A? As the mutation L143A in Lb^{pro} should lower the specificity of the protease, further cleavage products might be expected as the pocket of S₂ is able to accept other residues.

3 Materials and Methods

3.1 Bacterial culture

3.1.1 Bacterial strains

3.1.1.1 *E. coli* BL21 (DE3) pLysS (F- ompT hsdSB (rB-mB-) gal dcm (DE3) pLysS (CamR))

E. coli BL21 (DE3) pLysS (Novagen) was used to express pET11d eIF4GII, pET11d Stop Lb^{pro} wt, pET11d Lb^{pro} L143A and p_{pro}ExHTA – mElF4E. This strain is widely used for protein expression, mostly in combination with pET expression vectors. It is deficient in *lon* and *ompT* proteases. DE3 indicates that the strain contains a λ -DE3 and therefore carries a copy of the T7 bacteriophage RNA polymerase gene I under control of a lac promoter. The expression is induced by adding IPTG (Isopropyl β -D-1-thiogalactopyranoside) and the gene under control of the T7 promoter will be expressed. In addition, the pLysS plasmid includes the T7 lysozyme gene, which suppresses basal expression. BL21 (DE3) pLysS is mostly used to express cytotoxic proteins. The plasmid also contains a chloramphenicol resistance gene, 30 μ g/ml chloramphenicol were therefore added to the medium.

3.1.1.2 *E. coli* BL21(DE3)pLysE (F- ompT hsdSB (rB-mB-) gal dcm (DE3) pLysE (CamR))

For the expression of pET11d Lb^{pro}L143A the bacterial strain for expression was changed to *E. coli* BL21 (DE3) pLysE. BL21 (DE3) pLysE is similar to BL21 (DE3) pLysS and carries also the T7 lysozyme gene, but produces higher amounts T7 lysozyme to further reduce the basal level of expression.

3.1.1.3 *E. coli* Top 10F'

To modify or amplify plasmids, the *E. coli* Top 10F' strain (F-*mcrA* Δ (*mrr*-*hsdRMS-mcrBC*) Φ 80/*lacZ* Δ M15 Δ *lacX74* *recA1**araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* *endA1* *nupG*) from Invitrogen was used.

3.1.1.4 Media and Solutions

Luria Bertani (LB) medium (Roth) consists of 10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl, pH 7.0 $\pm$ 0.2. Before using the medium it was autoclaved. This medium was used to grow bacteria for DNA amplification or recombinant protein expression. For the latter additional antibiotics were added before use (ampicillin: 100 μ g/ml,

chloramphenicol: 30 µg/ml). LB-medium was used for protein expression of Lb^{pro} wt, Lb^{pro} L143A and eIF4GII.

Terrific Broth (TB) medium contains 12 g tryptone, 24 g yeast extract and 4 ml glycerol per liter of culture. Before using the sterile TB-medium, 100 ml of a solution containing 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄ were added as well as ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml). This medium was used to express eIF4E.

3.1.1.5 Preparation of competent cells

All three *E. coli* strains were made competent using a slightly modified protocol from D. Hanahan *et al.* "DNA Cloning Techniques". 3 ml of 2 x TY were inoculated with cells directly from the -80°C freezer and incubated overnight at 37°C and 220 rpm. The overnight culture was used to inoculate 100 ml of 2 x TY, which was kept at 37°C at 220 rpm until the OD₆₀₀ reached about 0.5. This culture was divided into two 50 ml falcons and incubated 15 min on ice. After harvesting the cells at 2500 rpm, 15 min at 4°C, the supernatant was discarded and the pellet resuspended in 33 ml of RF1. This was then again incubated for 15 min on ice, centrifuged at 2500 rpm for 15 min at 4°C. The pellet was resuspended in 8 ml of RF2 and 200 µl aliquots were snap frozen.

2 x TY:	16 g tryptone, 10 g yeast extract, 5 g NaCl, ad 1 L dH ₂ O
RF1:	100 mM KCl, 50 mM MnCl ₂ · 4 H ₂ O, 30 mM CH ₃ CO ₂ K, 10 mM CaCl ₂ · 2 H ₂ O, 15 % (w/v) glycerol, adjust to pH 5.8
RF2:	10 mM MOPS, 10 mM KCl, 75 mM CaCl ₂ · 2 H ₂ O, 15 % (w/v) glycerol, adjust to pH 6.8

3.1.1.6 Transformation of competent cells

All *E. coli* strains were used for transformation. Per transformation 200 µl of competent cells were thawed on ice for 15 min. Depending on the sample a certain amount (e.g. 10 µl for ligations, 1 µl for midi preps) was added to the cells and kept for another 15 min on ice. Samples were heat shocked at 42 °C for 45 seconds, 400 µl of preheated LB medium were added and then incubated at 37°C for at least 30 min. Between 150 and 200 µl were plated on LB- Amp Agar-Agar plates in the case of *E. coli* Top 10F' cells and on Lb-Amp-Cam Agar-Agar plates in the case of *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 (DE3) pLysE. Plates were incubated overnight at 37°C to allow colonies to grow.

LB-Amp Agar Agar plates: Add 1.5 % Agar-Agar to LB-Medium. Autoclave at 120 °C for 20 minutes. Let cool down, add ampicillin (100 mg/L) and pour plates.

3.2 DNA methods

3.2.1 Plasmids for protein expression

For the expression of recombinant proteins in either *E. coli* (DE3) pLysS or *E. coli* (DE3) pLysE, the vectors ρ proExHT a (Invitrogen) and pET11d (Novagen) were used. ρ proExHT a is 4779 bp in size and encodes sequences for T7 driven protein expression as well as the lacI gene. The vector carries additionally an ampicillin resistance selection marker. pET11d is 6165 bp in size and encodes the same regions as ρ proExHT a. The induction of protein expression is possible by the addition of IPTG.

3.2.2 Preparation of plasmid DNA from bacteria

3.2.2.1 DNA mini prep

Mini preps are small scale plasmid preparations of transformed *E. coli* cells to isolate and purify plasmid DNA (Birnboim and Doly 1979). These DNA preparations were used to screen for positive mutants.

4 ml of an LB-Amp culture were inoculated with a colony picked from a LB-Amp plate and. The samples were incubated at 37°C in a bacterial shaker overnight. The next day, cells were harvested in 1.5 ml tubes for two minutes at 6.000 rpm. After discarding the supernatant the spinning and discarding was repeated with the rest of the overnight culture. The pellet was resuspended in 100 μ l of Solution I and then mixed gently with Solution II. 150 μ l of Solution III were added and mixed again before centrifugation of the sample at 4°C for 10 minutes at 14.000 rpm. The supernatant was transferred into a new tube. For precipitation 1 ml of ice cold, 96 % ethanol was added and the tube inverted. After spinning down at 14.000 rpm for 5 min at 4°C, the pellet was taken up in 200 μ l TE-Buffer and 200 μ l of 5 M LiCl were added. The centrifugation was repeated before the supernatant was transferred into a new tube and the DNA precipitated with 1 ml of ice cold 96 % ethanol. The pellet received after centrifugation was dried on air for about 1 hour and the DNA taken up in 50 μ l MILI-Q water. Isolated and purified DNA was stored at - 20 °C.

Solution I:	50 mM Tris/HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNaseA
Solution II:	20 mM NaOH, 1 % SDS
Solution III:	2.8 M Potassiumacetate pH 5.1
TE-Buffer:	10 mM Tris/HCl pH 8.0, 1mM EDTA

3.2.2.2 DNA midi prep

Midi preparations are middle scale DNA preparations to produce larger amounts of clean DNA. The “Plasmid DNA Purification Kit” (Macherey-Nagel) was used to isolate DNA following the instructions of the company. The so purified plasmid DNA was taken up in 200 µl MILI-Q water and stored at - 20 °C. Those DNA preparations were used for sequencing and recombinant protein expression.

3.2.3 DNA quantification

DNA concentrations were quantified using the Nanodrop spectrophotometer ND-1000 from Peglab, following the instructions of the manufacturer.

3.2.4 Agarose gel electrophoresis

DNA gel electrophoresis was used to monitor the outcome of various reactions and procedures. The obtained DNA-fragments were separated on 1 % (w/v) agarose gels prepared with 0.5 x TAE buffer. 10 x loading buffer was added to the samples in the correct amount. The gels were run at 100-120 V using a power pack 300 from BioRad. 1 µg of *Hind*III digested λ-DNA was used as size marker. The DNA-fragments were visualized by a UV-transilluminator after staining with ethidiumbromide for 20-30 min.

0.5x TAE buffer:	20 mM Tris base, 5 mM sodiumacetate, 1 mM EDTA pH 8.2
10 x Loading Buffer:	1 mM EDTA, 0.1 % Orange G, 10 % Ficoll in 0.5 x TAE
Ethidiumbromide solution:	10 ⁻⁴ % ethidiumbromide in 0.5 x TAE

3.2.5 Restriction digestion of DNA

Preparative digestions of DNA were performed in a total volume of 20 μ l. Restriction endonucleases and 10 x reaction buffers from New England BioLabs were used.

Reaction:	x	μ l	DNA
	2	μ l	NEB buffer (1-4)
	0.3-1	μ l	enzyme
	y	μ l	dH ₂ O

The temperature and time of incubation was adapted to the enzymes chosen.

3.2.6 DNA dephosphorylation

To prevent religation of restricted plasmids the 5'-end of the DNA was dephosphorylated using calf-intestine alkaline phosphatase (CIP). After purification of the restricted vector using Wizard[®] SV Gel and PCR Clean-Up System (Promega) and elution in 44 μ l of dH₂O, 5 μ l of NEB buffer 3 (New England BioLabs) and 1 μ l of CIP (New England BioLabs, 10u/ μ l) were added. The reaction was incubated for 60 min at 37°C. After dephosphorylation, the vector was again cleaned.

3.2.7 Extraction of DNA from agarose gel

To separate a certain DNA band from others a preparative 1 % (w/v) agarose gel was performed (80 ml of 0.5 x TAE and 0.80 g agarose). After staining with ethidiumbromide and visualizing the DNA bands by UV light the desired band was cut out and purified using the Wizard[®] SV Gel and PCR Clean-Up System.

3.2.8 DNA ligation

Before ligation, 1 μ l of the dephosphorylated vector and 1 μ l of the insert were loaded on a 1 % agarose gel to estimate their concentration. For ligation a ratio of 1:3 of vector to insert was used. The ligation itself was performed at room temperature for 3 hours.

	x	μ l	vector
	y	μ l	insert
	2	μ l	10 x ligase buffer (Promega)
	0.5	μ l	T4 DNA ligase (Promega)
	z	μ l	dH ₂ O
total of:	20	μ l	

3.2.9 DNA purification

3.2.9.1 Wizard® SV Gel and PCR Clean-Up System (Promega)

The Wizard® SV Gel and PCR Clean-Up System was used throughout the work to extract DNA from preparative agarose gels as well as for cleaning DNA (e.g. after PCR, dephosphorylation, restriction etc.).

To extract DNA from a gel, the desired band was cut out and transferred into an eppendorf tube. An equal amount of membrane binding solution was added and heated at 60 °C until the gel slice was completely dissolved. To clean DNA, an equal volume of membrane binding solution was added. Further steps were done similar for both reactions.

The SV minicolumn was inserted into the collection tube and either the dissolved gel mixture or the prepared PCR sample were transferred onto the minicolumn, incubated at room temperature for 1 min and centrifuged at 14000 rpm for 1 min. The flow-through was discarded and the column washed once with 700 µl and then with 500 µl membrane wash solution. Centrifugation was done at 14.000 rpm for 1 and 5 min respectively. The collection tube was sucked dry with a vacuum pump and the minicolumn was reinserted and centrifuged again for 5 min at 14.000 rpm. To elute the DNA sample, the minicolumn was transferred into an eppendorf tube and 30 – 50 µl of dH₂O were added. After 1 min of incubation at room temperature, centrifugation was repeated at 14.000 rpm for 1 min. Cleaned DNA was stored at - 20 °C.

3.2.10 DNA sequencing

DNA sequencing was performed by LGC Genomics (<http://www.agowa.de/>). For sequencing of pET11d Lb^{pro} L143A, the primers T7 promotor and T7 terminator provided by the sequencing service were used. Plasmids were sent in 10 µl aliquot of 100 ng/µl.

3.3 Protein methods

3.3.1 Protein Expression in LB and TB media

The expression of recombinant protein was accomplished in *E. coli* BL21 (DE3) pLysS or *E. coli* BL21 (DE3) pLysE using. Bacteria were transformed with the vector containing the cDNA coding for the desired protein. Then 100 ml of LB-Amp-Cam (100 µg/ml and 30 µg/ml respectively) medium were inoculated with a single colony carrying the desired construct and incubated overnight at 37°C on the shaker. The overnight culture was diluted up to 1 L of LB-Amp-Cam and grown at 32 °C. Once the optical

density (OD_{600}) of 0.6 was reached, protein expression was induced by adding IPTG. Before induction 25 ml were taken into a separate flask to monitor the growth without IPTG (uninduced fraction). The induced and the uninduced culture were then kept for a certain time at a certain temperature depending on the protein. After expression, cells were harvested by centrifugation at 5000 rpm for 15 min at 4°C. The pellets of the induced and uninduced fraction were resuspended in suitable lysis buffer and both stored at - 80 °C.

Luria Broth medium: 10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl pH 7.0 ± 0.2

Teriffic Broth medium: 12 g/l tryptone, 24 g/l yeast extract, 4 ml/l glycerol autoclave and then add 0.17 M KH_2PO_4 and 0.72M K_2HPO_4

3.3.2 Preparation of *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 (DE3) pLyse cell extracts

To disrupt the cells, the resuspended bacterial pellets were thawed on ice and sonicated with the homogenizer Sonoplus HD 200 from Bandelin in combination with the probe tip MS 73D. The induced fractions were sonicated three times 30 sec at a cycle of 40% and finally one continuous cycle for 15 sec. For the uninduced fraction, sonication was performed three times 30 sec at a cycle of 20%. The tubes were kept on ice.

1 ml of the induced fraction was taken into a clean eppendorf tube; the remaining 29 ml were centrifuged at 18.000 rpm for 30 min at 4°C. 100 µl of the 1 ml aliquot of the induced fraction and of the uninduced fraction were taken as “total” sample for a SDS PAGE. The remaining was centrifuged at 13.000 rpm for 30 min at 4°C to separate the soluble fraction (supernatant) from the insoluble one (pellet). Aliquots of the soluble, insoluble and total sample were then investigated on a SDS PAGE.

The supernatant of the induced fraction was used for further protein purification steps (see 3.3.4).

3.3.3 Preparation of HeLa S10 extracts

HeLa S10 cell extracts were used to test the activity of Lb^{pro} wt and Lb^{pro} L143A as described in 3.3.13. First 1 l of HeLa cells were grown in suspension culture and harvested in log phase at 1.200 rpm, 10 min at 4°C (Beckmann- J6-HC centrifuge). After discarding the medium, the cells were washed once with 50 ml ice cold 1 x PBS and twice with 25 ml ice cold 1 x PBS. Between each washing step, centrifugation at 1.000

rpm for 8 min at 4°C was performed (Heraeus Megafuge 1.0 R). The pellet was resuspended in 1.5 volumes of hypotonic lysis buffer and allowed to swell on ice for 10 min. Subsequently, cells were homogenized with 250 to 300 strokes in a suitable Wheaton Dounce homogenizer and the disruption of cells controlled with trypan blue, which colors selectively dead cells. To remove cell debris and nuclei, the extract was transferred to a 30 ml glass centrifuge tube (Correx) and centrifuged for 5 min at 3000 rpm at 4°C (Sorvall HB-4 rotor). At increased rpms (13.000 for 20 min) mitochondria was removed as well. After dialysis for 2 to 3 hours at 4°C against 1 l of dialysis buffer, the cell extract was centrifuged in glass tubes at 13.000 rpm for 10 min at 4°C. Prior to aliquoting and snap-freezing, the upper white layer needs to be removed. The samples were stored at - 80 °C.

Hypotonic lysis buffer: 10 mM HEPES adjust pH 7.6 with KOH, 10 mM
CH₃CO₂K, sterile filtered and stored at 4°C
add 2 mM DTT before use

Dialysis buffer: 10 mM HEPES adjust pH 7.2 with KOH, 90 mM
CH₃CO₂K, sterile filtered and stored at 4°C
add 1 mM DTT before use

3.3.4 Protein purification

3.3.4.1 Fractionated ammonium sulfate precipitation

Ammonium sulfate precipitation is a method to purify the protein by changing its solubility. In the first step of the fractionated ammonium sulfate precipitation 30 % (v/v) of saturated ammonium sulfate were added to the soluble fraction. This was kept stirring at 4°C overnight. After centrifugation at 18.000 rpm for 30 min at 4°C, saturated ammonium sulfate up to 60 % (v/v) were added to the supernatant and stirred 4 h at 4°C. The solution was centrifuged at 11.000 rpm for 30 min at 4°C, and the pellet resuspended in 4 ml Buffer B (per 1 l of culture).

Buffer B: 50 mM Tris/HCl pH 8.0, 1 M NaCl, 1 mM EDTA, 5 mM DTT, 5 %
(w/v) glycerol

3.3.4.2 Dialysis

To remove the excess of salt, dialysis against buffer B was performed. Hence, the protein solution was transferred into a dialysis membrane made of cellulose (Snake Skin™ Pleated Dialysis Tubing from Thermo Scientific) with a cut-off of 7 kDa. The bags

were placed in 500 ml Buffer B. The Buffer was changed twice, after 1.5 hours and after 3 hours. Finally, the sample was dialyzed against 800 ml of buffer B overnight. Dialysis was performed on 4°C.

3.3.4.3 FPLC

Subsequently, the protein was purified further on an ÄKTA Fast Protein Liquid Chromatography (FPLC) system from Amersham Biosciences. Depending on the protein either anion-exchange chromatography (MonoQ 10/100 GL) or affinity chromatography (HisTrap HP, 1ml) was performed in the first purification step. Further purification was obtained using size-exclusion chromatography (HiLoad 26/60 Superdex 75 pg). Columns used are from GE Healthcare.

3.3.4.3.1 Anion exchange chromatography

Ion exchange chromatography retains proteins based on their ionic interactions. Anion exchange chromatography especially retains negatively charged proteins, which bind to positively charged functional groups on the stationary phase of the column.

MonoQ 10/100 GL was used for the purification of Lb^{pro} wt, Lb^{pro} L143A and the eIF4E. Prior to loading the sample, the pumps and the loop were washed with filtered dH₂O (column on bypass). Then the column was washed with two column volumes (CV) of filtered dH₂O and equilibrated with 2 CV of the loading buffer (Buffer A). The filtered sample was loaded via a 10 ml loop or a 50 ml superloop onto the column. Depending on the protein the gradient was chosen (table 1). UV light at 280 nm was used for detection of the eluted fractions. 2 ml fractions were collected automatically. After the run the column was washed with filtered dH₂O, 20 % ethanol and stored at room temperature.

	Lb ^{pro} wt	Lb ^{pro} L143A	eIF4E fragment
Buffer A	50 mM Tris HCl 8, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, 5 % (v/v) glycerol		
Buffer B	50 mM Tris HCl 8, 1 M NaCl, 1mM EDTA, 5 mM DTT, 5 % (v/v) glycerol		
gradient	5 column volumes (CVs) of 100% Buffer A, 2.5 CVs of 0-30 % Buffer B, 15 CVs of 30-60 % Buffer B, 2 CVs of 60-100 % Buffer B and 5 CVs of 100 % Buffer B		2 CVs of 100% Buffer A, 10 CVs of 0-30 % Buffer B, 6 CVs of 30-60 % Buffer B, 2.5 CVs of 60-100 % Buffer B and 1 CV 100 % Buffer B

Table 1: Gradient and buffers used for MonoQ 10/100 GL.

3.3.4.3.2 Size-exclusion chromatography

Proteins in solution can be separated according to their size via size-exclusion chromatography. Throughout this work a HiLoad 26/60 Superdex 75 pg column was used for purification. The different proteins were always separated over 1.2 CVs of Buffer A. UV light at 280 nm was used for detection of the eluted fractions. 2 ml fractions were collected automatically.

3.3.4.3.3 Affinity chromatography

3.3.4.3.3.1 HisTrap HP

The purification of tagged proteins is based on the interactions between the tag and immobilized ligands on the column. eIF4GII is tagged with histidine. Histidines tagged proteins show a high affinity for Ni^{2+} ions and are therefore selectively bound to immobilized Ni^{2+} ions on a column. This property is used for separation.

To purify eIF4GII a 1 ml HisTrap HP column was used. Before use the column was washed with dH_2O . To charge the column with Ni^{2+} ions, 5 ml 500 mM NiCl_2 were loaded onto the column. After equilibration with HisTrap Binding Buffer, the sample was loaded via a 10 ml loop. To separate eIF4GII either a one step gradient (8 CV of 0 %, 30 CV of 0 – 100% and 10 CV of 100 % HisTrap elution buffer) or a two step gradient (2 CV of 0 %, 12 CV of 0 – 35 %, 8 CV of 35 – 100 % and 10 CV of 100 % HisTrap elution buffer) were used. UV light at 280 nm was used for detection of the eluted fractions. Fractions of 1ml were collected and stored at 4°C.

HisTrap binding buffer:	50 mM Tris/HCl pH 8.0, 50 mM NaCl, 10 mM imidazole
HisTrap elution buffer:	50 mM Tris/HCl pH 8.0, 50 mM NaCl, 500 mM imidazole

3.3.4.3.3.2 Binding to 7-Methyl-GTP Sepharose 4B beads

eIF4E samples were first purified with MonoQ 10/100 GL (see 3.3.5.1). Fractions containing eIF4E were pooled and concentrated before dialysis against eIF4E binding buffer at 4°C. The next day 500 µl of eIF4E were incubated overnight at 4°C with 1 ml of 7-Methyl-GTP Sepharose 4B beads (GE Healthcare). The unbound fractions were washed off with 6 ml eIF4E binding buffer and the beads incubated 3 h at 4°C with 600 µl eIF4E elution buffer. eIF4E was eluted with 10 ml eIF4E elution buffer and the beads were washed with 4 ml of the same buffer. For regeneration, the beads were washed with 20 ml dH_2O , 20 ml 0.2 % SDS, again 20 ml dH_2O and 20 ml 0.02 % sodium azide

and stored at 4°C. All wash and elution fractions were collected in 2 ml and 1 ml respectively.

eIF4E binding buffer: 20 mM MOPS pH 7.6, 0.25 mM DTT, 0.1 mM EDTA, 50 mM NaF, 100 mM KCl

eIF4E elution buffer: 20 mM MOPS pH 7.6, 0.25 mM DTT, 0.1 mM EDTA, 50 mM NaF, 500 mM KCl

3.3.5 Protein concentration

Protein samples were concentrated using Centrprep Centrifugal Filter Devices (Millipore) following the instructions of the manufacturer. The centrifugations were performed at 3.000 rpm at 4°C on a Beckman J6-MI centrifuge.

3.3.6 Protein quantification

Besides controlling the concentration of proteins by SDS PAGE, the Nanodrop spectrophotometer ND-1000 from PeggLab was used according to the instructions of the manufacturer.

3.3.7 SDS PAGE (Sodiumdodecylsulfate Polyacrylamide Gel electrophoresis) (Laemmli, 1970)

SDS PAGE was performed according to the protocol of Laemmli 1970. For the gel electrophoresis the mini-PROTEAN® 3 system from BioRad was used and assembled according to the instructions. The composition of the gel is shown in table 2. The components of the separation gel were mixed whereupon TEMED was added last. The mix was then poured and overlaid with isopropanol. After polymerization, the gel was rinsed with water, the stacking gel mixture poured on top of it and a comb placed in the gel. The gel was arranged in a tank after polymerization and the tank filled with Laemmli running buffer. A certain amount of protein fractions were mixed with either 2 x or 5 x Laemmli sample buffer and heated at 95 °C for 10 min. The obtained samples were loaded on the gel and protein separation was achieved at 100-150 V using an electrophoresis power supply from BioRad. 7 µl Prestained Precision Plus Protein standards from BioRad were used as size marker.

	Separation gel		Stacking gel (ml)
	17.5 % (ml)	6 % (ml)	
30 % Polyacrylamide (PAA)	3.500	1.200	0.333
2.5 % Bisacrylamide (BAA)	0.209	0.209	0.104
4 x Lower gel solution (LGS)	1.500	1.500	-
4 x Upper gel solution (UGS)	-	-	0.500
dH ₂ O	0.758	3.060	1.060
10 % Ammoniumperoxidsulfate (APS)	0.050	0.050	0.020
Tetramethylethylenediamine (TEMED)	0.005	0.005	0.002

Table 2: Composition of "Laemmli" separation gel and stacking gel.

LGS:	1.5 M Tris base pH 8.8, 0.4% SDS
UGS:	0.5 M Tris base pH 6.8, 0.4% SDS
2 x Laemmli sample buffer:	20 % glycerol, 10 % β -mercaptoethanol, 6 % SDS, 125 mM Tris, 0.01 % Bromphenol Blue pH 6.8
5 x Laemmli sample buffer:	50% glycerol, 25 % β -Mercaptoethanol, 15 % SDS, 0.31 M Tris, 5 mg Bromphenol Blue, pH 6.8
Laemmli running buffer:	25 mM Tris base, 0.2 M Glycine, 0.1 % (w/v) SDS

3.3.8 Coomassie Staining of Polyacrylamide Gels

The polyacrylamide gels were stained by incubation with coomassie staining buffer for about 20-30 min. Destaining of the gels occurred in lukewarm water overnight at room temperature. After destaining, the gels were vacuum-dried on Whatman TM 3MM paper for 1 hour at 80 °C using a Slab Gel Dryer SGD 4050 from Savant.

Coommassie staining buffer:	0.4 % (w/v) BioRad Coomassie Brilliant Blue R250, 45 % (v/v) methanol, 10 % (v/v) acetic acid
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3.3.9 Western Blot

For western blot analysis the protein samples and the Prestained Precision Protein marker were first separated by SDS PAGE. The proteins were then transferred to a PVDF ImmobilonTM-P Transfer Membrane, which was activated with methanol before use. The western blot sandwich consisted of 2 sponges and 3 pieces of WhatmanTM 3MM paper soaked with transfer buffer which enclose the SDS gel and the transfer membrane. This was then put into a TE 22 Mini Transfer Tank Unit from Hoefer scientific instruments. Prior to blotting the tank was filled with transfer buffer and blotting was performed at 4°C either overnight at 40 mA or at 200 mA for 3 h.

To analyze samples containing eIF4GII (either purified or from HeLa cell extract) the following procedure was performed:

For the immunoblot the transfer membrane was incubated with 5 ml blocking buffer for 30 min in a falcon tube at room temperature rolling to block unspecific binding sites. Subsequent, the membrane was incubated with the primary antibody (table 3) for 1 hour at room temperature. The membrane was washed two times 10 min with 1 x PBS (Phosphate buffered saline) and 5 ml of the secondary antibody in 1 x PBS were added for 1 h at room temperature. Before detection, the membrane was washed three times 10 min with 1 x PBS. In the case of alkaline phosphatase (AP) conjugated secondary antibodies, the membrane was incubated with 5 ml of alkaline phosphatase buffer, 25 μ l of freshly made 5 % NBT and 25 μ l of 2.5 % BCIP. Once proteins were visible (1-15 min), the reaction was stopped with dH₂O.

For the detection of histidine tagged proteins, the membrane was incubated with 5 ml blocking buffer for 30 min at 4°C. Thereafter, additional 5 ml blocking buffer containing 1 μ l of His Probe (final dilution of 1:10.000) were added to the membrane and incubated 1 h at 4°C. Before detection, the membrane was washed 3 times with 1 x PBST and 2 x with 1 x PBS. For horseradish peroxidase (HPR) conjugated antibodies, the Super Signal® West Pico chemiluminescent substrate kit from Pierce was used. Therefore, the membrane was incubated for a few minutes with 1 ml of each of the two solutions and then wrapped into clingfilm. The membrane was exposed to an autoradiography film (BioMax MS film from Kodak).

Transfer buffer:	1 % (w/v) glycine, 20 % (v/v) methanol, 25 mM Tris base pH 8.8
Blocking buffer:	0.2 % Tween 20, 0.2 % I-Block (Tropix) in PBS
PBS(T):	1.4 mM KH ₂ PO ₄ , 2.7 mM KCl, 4.3 mM Na ₂ HPO ₄ , 137 mM NaCl, (0.1 % Tween 20)
Alkaline phopshatase:	5 mM MgCl ₂ , 100 mM NaCl, 100 mM Tris HCl pH 9.6
5% NBT:	5 % (w/v) nitro blue tetrazolium chloride in 90 % dimethylformamide
2.5 % BCIP:	2.5 % 5-bromo-4-chloro-3-indolyl phosphate in dH ₂ O

Primary antibodies	dilution
rabbit anti eIF4GI (N-terminal, antiserum, gift from R.Rhoads, Shreveport, LA)	1:8.000
rabbit anti Leader protease (antiserum)	1: 2.000
His Probe TM horseradish peroxidase conjugated (Pierce)	1:10.000

Secondary antibody	dilution
anti rabbit IgG alkaline phosphatase coupled (Sigma)	1:5.000

Table 3: List of primary and secondary antibodies used in this work for immunodetection.

3.3.10 Blue Native PAGE (BN PAGE)

Blue native PAGE was used to analyze proteins in their native state. It was developed to separate mitochondrial membrane proteins and complexes of 10 kDa to 10.000 kDa (Ilka Wittig *et al.* 2006, Schägger and von Jagow 1991). The BN PAGE uses Coomassie blue G250 dye which provides the negative charges for the surface of the protein for the electrophoretic separation. Native proteins and complexes migrate to the anode as blue bands and are separated according to their molecular weight and/or size as in SDS PAGE.

The solution for the sample gel is mixed out of the two stock solutions of 5 % and 18 % acrylamide depending on the percentage of choice. Usually a gradient gel is used, but in this work either 10 or 15 % sample gels and 5 % stacking gels were used. Vertical gels are casted in a cassette out of two glass plates separated by spacers. After polymerization of the sample gel, covered with a layer of dH²O, the stacking gel is casted and the well comb inserted. The electrophoresis apparatus is assembled in the cold room and the water cooling turned on. After the removal of the comb the blue cathode buffer is poured into the upper chamber. Prior to loading, the samples were supplemented with ¼ volume of sample buffer and kept on ice. Following the loading of the samples, the anode buffer was poured into the lower buffer chamber. As soon as the power supply is connected to the electrophoresis unit, the electrophoretic run is started at 100 V overnight.

When the blue dye front reached the half of the gel, the blue cathode buffer is replaced with the colorless cathode buffer and the run continued with maximal 400 V. Once the blue cathode buffer front reached the bottom of the gel, the run is stopped.

Proteins can then be visualized by staining with Coomassie between 3 h-overnight. After destaining, the gel can be kept in the destaining solution at 4°C or dried.

	sample gel	5 %	15 %
AB mix	0.5 ml	18 ml	35 ml
Gel Buffer 3 x	2 ml	60 ml	50 ml
Glycerin (87 %)	-	-	24.4 ml
dH ₂ O		101.9 ml	20.05 ml
10 % APS	50 µl		
TEMED	5 µl		

Table 4: Composition of 10 x stock solutions of 5 % and 18 % acrylamide.

For 1 gel:	17.89 ml 5 %, 100 µl APS (10 %), 10 µl TEMED 14.945 ml 18 %, 50 µl APS (10%), 5 µl TEMED
AB mix:	48 g acrylamide, 1.5 g bisacrylamide in 100 ml dH ₂ O
Cathode Buffer:	50 mM Tricine, 15 mM Bistris pH 7.0, 0.02 % Coomassie brilliant blue G250
Cathode Buffer 2:	50 mM Tricine, 15 mM Bistris pH 7.0
Anode Buffer:	50 mM Bistris, adjust pH 7.0 with HCl
Gel Buffer (3x):	1.5 M 6-aminocaproic acid, 150 mM Bistris pH 7.0
Sample Buffer:	500 mM aminocaproic acid, 5 % Coomassie brilliant blue G250
Staining solution:	125 mg Coomassie brilliant blue G250, 200 ml ethanol, 35 ml acetic acid, ad 500 ml dH ₂ O
Destaining solution:	20 % methanol, 7.5 % acetic acid

3.3.11 Activity assays with HeLa cell extracts

Activity assays of HeLa cell extracts were performed with either Lb^{pro} wt or Lb^{pro} L143A. HeLa cell extracts contain eIF4GII, which is cleaved by all picornaviral proteases.

8 – 10 µl of HeLa cell extracts were incubated with different amounts of Lb^{pro} wt or Lb^{pro} L143A for 60 min at 30°C. The reaction was stopped with 5 x sample buffer, heated at 95 °C for 10 min and loaded on a 6 % SDS PAGE. After electrophoresis and western blot analysis, detection was performed as described in 3.3.10.

3.3.12 Processing assays with purified eIF4GII or eIF4GII^{self}

To test the different cleavage properties of Lb^{pro} wt and Lb^{pro} L143A, the processing was observed on the purified eIF4GII fragment or eIF4GII^{self}. For this purpose 1 µl of the substrate was incubated at 30°C for various times with different amounts of either Lb^{pro} wt or Lb^{pro} L143A. The reaction was stopped by adding 5x sample buffer, and the proteins separated on by 17.5% SDS PAGE. The gels were stained with Coomassie staining buffer for about 30 min and destained in lukewarm water overnight.

In some processing assays eIF4E was preincubated with either eIF4GII or eIF4GII^{self} overnight at 4°C before the processing with the protease.

3.3.13 MALDI-TOF Mass Spectrometry analysis

MALDI-TOF MS (Matrix assisted laser desorption ionization – time of flight mass spectrometry) is used to identify proteins and peptides. The protein samples were first separated by SDS-PAGE and coomassie stained. After destaining, the desired protein band was cut out under lamina flow and the gel slice kept on ice in SDS running buffer. Before the measurements, the sample was enzymatically digested with trypsin. Trypsin is used very frequently, because each proteolytic fragment contains a basic arginine or lysine amino acid residue and therefore is suitable for positive ionization mass spectrometric analysis. The digested sample is analyzed without further clean up steps. The spectrum displays the molecular weight from the proteolytic fragments. This so called peptide map, is compared to theoretical mass values in databases (non-redundant database and a small internal database). If the protein exists in a database, the peptide map is sufficient to identify the protein. The measurements were performed at the Mass Spectrometry Facility of the Max F. Perutz Laboratories headed by Professor Gustav Ammerer.

4 Results

4.1 Construction of Lb^{pro} L143A

The vector pET11d Lb^{pro} L143A (figure 17) used to express Lb^{pro} L143A was created by digesting the already available vectors pCITE Lb^{pro} L143A L200F and pET11d Lb^{pro} wt.

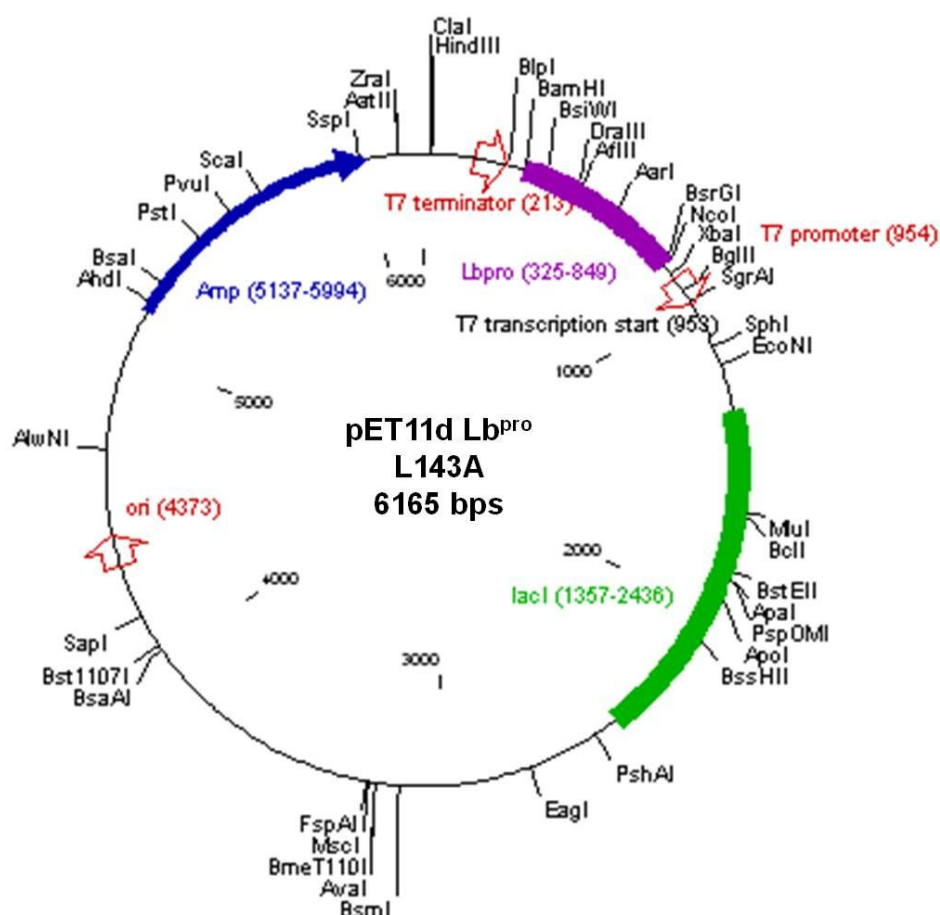


Figure 17: Vector map of pET11d Lb^{pro} L143A. The ampicillin resistance gene (Amp) and the *lacI* gene coding for the *lac* repressor protein are shown in blue and green, respectively. The gene of interest (Lb^{pro} L143A) is displayed in magenta flanked by the T7 terminator and T7 promoter. The origin of replication (ori) and the T7 terminator and promoter are shown as red arrows.

Both plasmids were cut with *NcoI* and *BsiWI*. The pCITE vector was cut in a way that only the L143A mutation remained. In contrast, the pET11d plasmid cut with the same enzymes, functions as vector (3.2.5). After the ligation of 2 μ l vector and 2 μ l insert for 2 hours at room temperature, 10 μ l of the ligation were transformed into *E. coli* Top 10F' cells and plated on a LB-Amp plate (3.2.8). The plate was incubated overnight at 37°C. The ligation was examined by digestion of minipreps with 0.3 μ l *NcoI* and 0.3 μ l *BsiWI* as a control before sequencing (3.2.10). Before using the plasmid for expression, midi preparations were done (3.2.2).

4.2 Expression and Purification

4.2.1 Lb^{pro} L143A and Lb^{pro} wild-type

Lb^{pro} wt and Lb^{pro} L143A, were expressed alike using *E. coli* BL21(DE3)pLysE cells. 2L of LB-Amp-Cam culture were used and the expression was induced with 0.3 mM IPTG at an OD₆₀₀ between 0.5 - 0.6. The protein was expressed at 16°C overnight (3.3.1). In both cases, the protease was not distinguishable from other proteins of similar size directly after expression and sonication. Figure 18 shows the 17.5% SDS gels of the soluble, insoluble and total fraction after sonication of Lb^{pro} L143A (a) and Lb^{pro} wt (b).

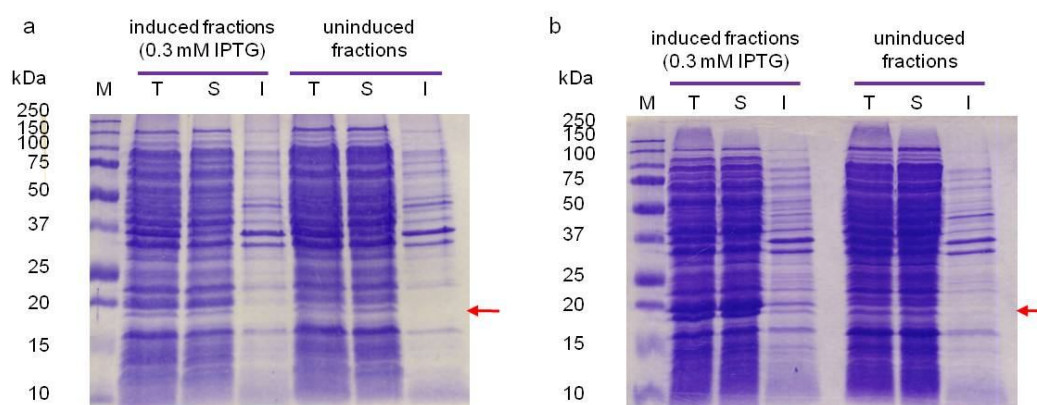


Figure 18: Total (T), soluble (S) and insoluble (I) fractions of (a) Lb^{pro} L143A and (b) Lb^{pro} wt after sonication. Expressions of each protease were done in 2 L of LB-Amp-Cam culture using *E. coli* BL21(DE3)pLysE. 0.3 mM IPTG were used to induce each culture prior to overnight expression at 16°C. No IPTG was added to the uninduced fractions. 9 µl of the induced fractions (total of 60 ml) and 9 µl of the uninduced fractions (total of 1 ml) were loaded. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

Subsequently, purification steps included the stepwise precipitation with saturated ammonium sulfate, purification by anion-exchange and size-exclusion chromatography (3.3.4).

The chromatogram of the separation of Lb^{pro} L143A by anion-exchange chromatography using the column MonoQ 10/100 GL is shown in figure 19a. A three step gradient of Buffer B was used for the separation. The 2 ml fractions A6-B4 were analyzed on a 17.5% SDS gel (figure 19b). The fractions containing Lb^{pro} L143A, A10-A14 and A15-B4, were pooled and concentrated up to 1.5 and 1 ml, respectively. Both pools were used for subsequent size-exclusion chromatography.

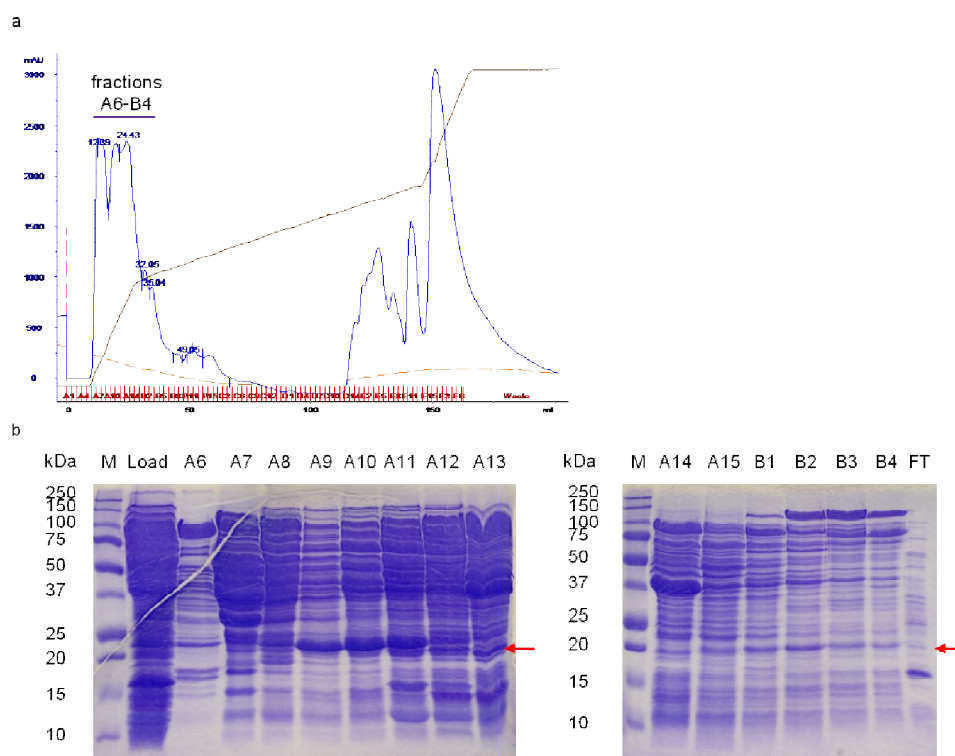


Figure 19: Purification of Lb^{pro} L143A by anion-exchange chromatography.

(a) Chromatogram of Lb^{pro} L143A purified by anion-exchange chromatography using a MonoQ 10/100 GL column. 16 ml of the Lb^{pro} L143A sample expressed in 2L of culture were loaded after stepwise precipitation. Fractions analyzed by SDS PAGE are indicated. **(b)** 17.5% SDS gel of fractions collected during anion-exchange chromatography. 9 μ l of each fraction (total of 2 ml) and the loaded protein (Load- total of 16 ml) are present on the gel. Fractions A15-B4 were pooled and concentrated prior to separation by size-exclusion chromatography. The brown line represents the conductivity. The red arrow points at Lb^{pro} L143A running at about 19 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

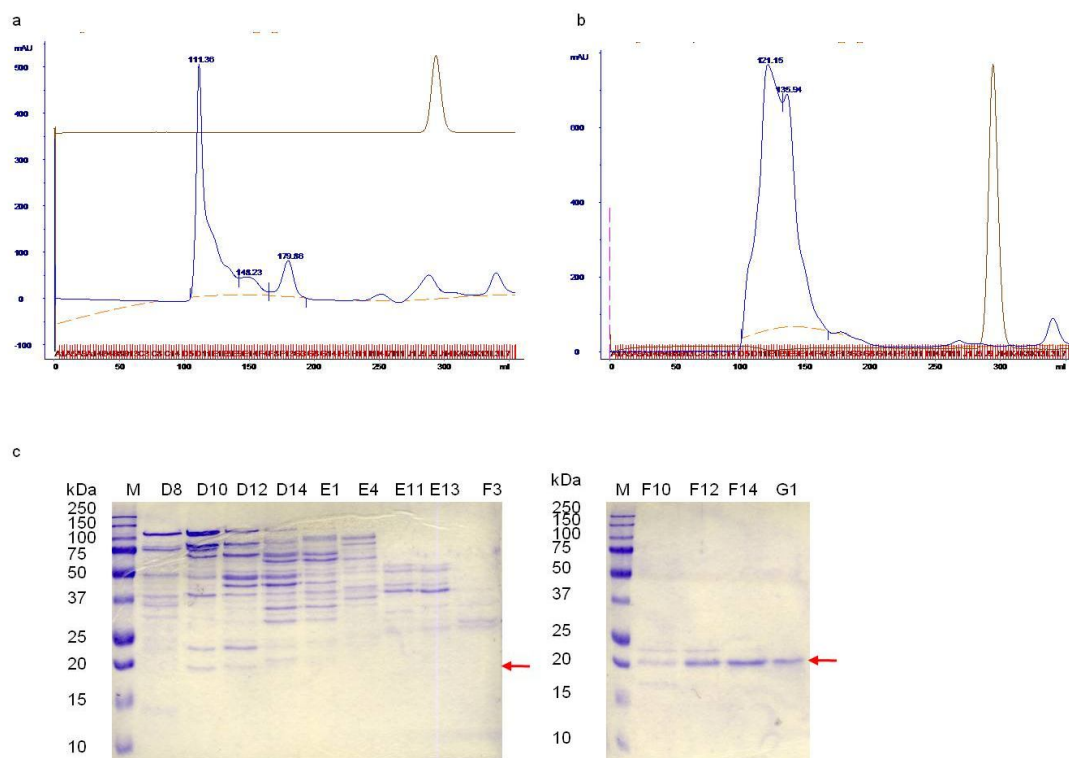


Figure 20: Purification of Lb^{pro} L143A by size-exclusion chromatography. (a) Chromatogram of Lb^{pro} L143A purified by size-exclusion chromatography using a HiLoad 26/60 Superdex 75 pg. Lb^{pro} L143A sample expressed in 2L of medium. Pooled fractions A15-B4 (1ml) of anion-exchange chromatography were loaded. (b) Chromatogram of Lb^{pro} L143A purified by size-exclusion chromatography using a HiLoad 26/60 Superdex 75 pg. Lb^{pro} L143A sample expressed in 2L of medium. Pooled fractions A10-A14 (1.5 ml) of anion-exchange chromatography were loaded. The brown curve represented in the chromatograms displays the conductivity. (c) 17.5% SDS gel of fractions collected during size-exclusion chromatography shown in (a). 9 µl of each fraction (total of 2 ml) are present on the gel. Fractions F10-F13 and F14-G1 were pooled and each concentrated up to 1.5 ml. The red arrow points at Lb^{pro} L143A running at about 19 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

Lb^{pro} wt was purified similarly. After stepwise precipitation with ammonium sulfate, the sample was loaded onto a MonoQ 10/100 GL column and 2 ml fractions collected using a three step gradient of Buffer B (3.3.4). The chromatogram is shown in Figure 21a. Fractions A15-C6 were examined by 17.5% SDS gel (figure 21b) and subsequently fractions B3-B8 were pooled and concentrated up to 1 ml for further size-exclusion chromatography, using a HiLoad 26/60 Superdex 75 pg column.

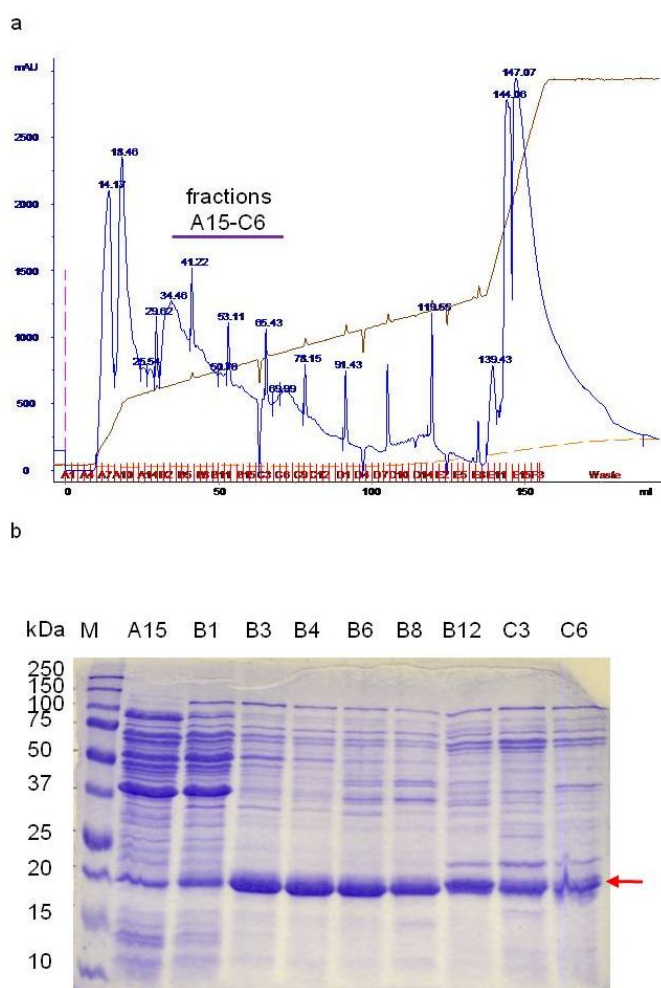


Figure 21: Purification of Lb^{pro} wt by anion-exchange chromatography. (a) Chromatogram of Lb^{pro} wt purified by anion-exchange chromatography using a MonoQ 10/100 GL column. 20 ml of the Lb^{pro} wt sample expressed in 2L of culture were loaded after stepwise precipitation. The brown line represents the conductivity. Fractions analyzed by SDS PAGE are indicated. (b) 17.5% SDS gel of fractions collected during anion-exchange chromatography. 9 μ l of each fraction (total of 2 ml) are present on the gel. Fractions B3-B8 were pooled and concentrated prior to separation by size-exclusion chromatography. The red arrow points at Lb^{pro} wt running at about 19 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

The chromatogram of Lb^{pro} wt separated by size-exclusion chromatography is shown in figure 22a. The peak at fractions A1-A3 and fractions C3-C14 were analyzed by 17.5% SDS gel (figure 22b). Fractions containing Lb^{pro} wt, C7-C14 were pooled and concentrated up to 600 μ l before use in subsequent experiments.

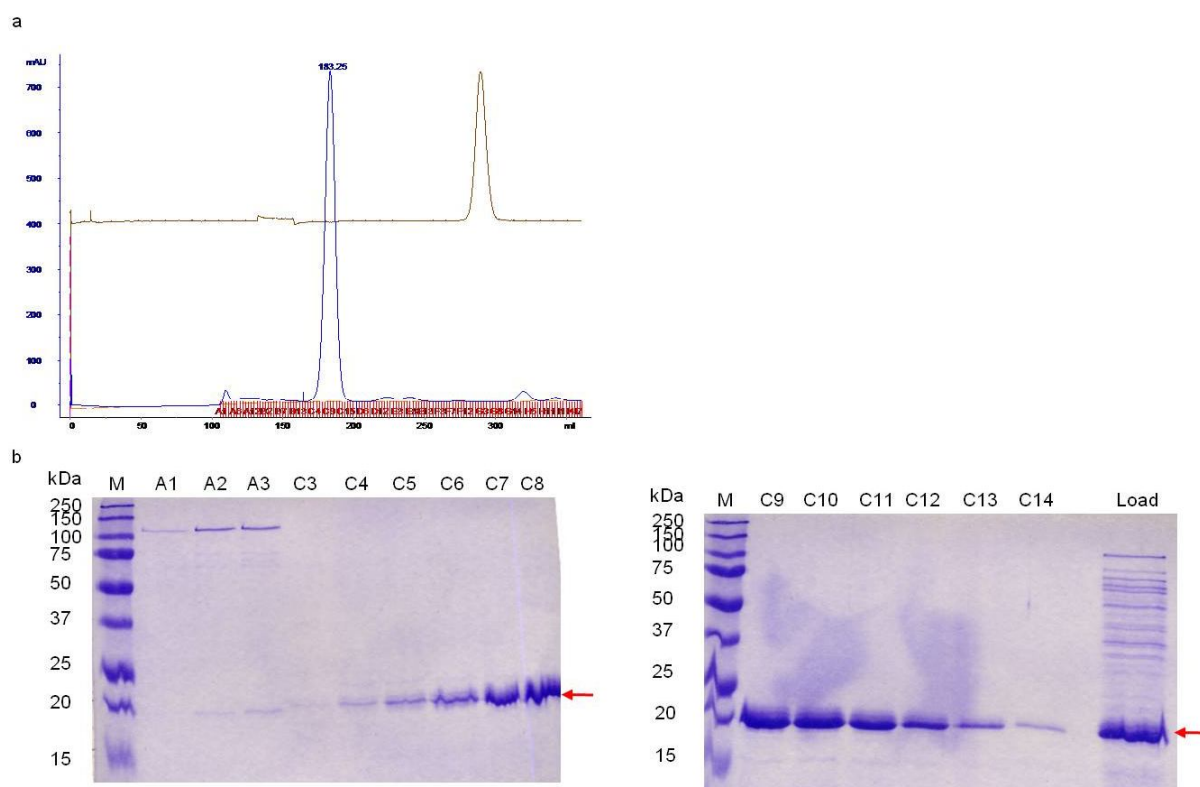


Figure 22: Purification of Lb^{pro} wt by size-exclusion chromatography. (a) Chromatogram of Lb^{pro} wt purified by size-exclusion chromatography using a HiLoad 26/60 Superdex 75 pg. Lb^{pro} wt sample of an expression in 2L of medium. Pooled fractions B3-B8 of anion exchange-chromatography (1ml) were loaded. The brown line represents the conductivity. (b) 17.5% SDS gel of fractions A1-A3 and C3-C14 collected during size-exclusion chromatography and of the loaded sample (Load). 9 μ l of each fraction (total of 2 ml) and 1 μ l of the loaded sample are present on the gel. Fractions C7-C14 were pooled and concentrated up to 600 μ l. The red arrow points at of Lb^{pro} wt running at about 19 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

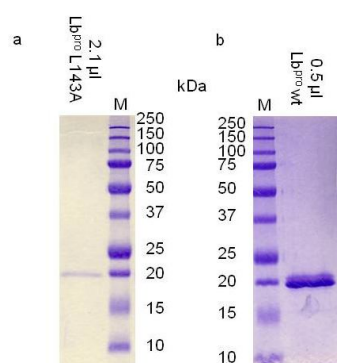


Figure 23: Purified (a) Lb^{pro} L143A and (b) Lb^{pro} wt. 0.21 μ g of Lb^{pro} L143A and 5 μ g of Lb^{pro} wt are present on the gel. The indicated amount was loaded on a 17.5% SDS gel. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

Even though the expression and purification methods were equivalent the yield was quite different. Both proteases were expressed using 2L of culture. However, the concentration of Lb^{pro} wt was 10 μ g/ μ l and of Lb^{pro} L143A only 0.1 μ g/ μ l. Figure 23 shows the purified and concentrated samples used in the following processing assays.

4.2.2 eIF4GII and eIF4GII^{self}

During my work, two different eIF4GII fragments were used, termed eIF4GII and eIF4GII^{self} (figure 24). Both comprise amino acids 552-745 of human eIF4GII (accession number: AF012072) and are theoretically 22 kDa in size, but were observed at 25 kDa on 17.5% SDS gels. For affinity purification and detection by western blot analysis, six histidine residues were added to the C-terminus of both substrates. Both fragments contain the eIF4E binding sequence (green in figure 24) and the related sequence not recognized by Lb^{pro} wt (underlined in figure 24). eIF4GII contains the natural cleavage sequence recognized by Lb^{pro} wt (LLNVG↓SRRSQ), whereas in eIF4GII^{self} (cloning performed by Katharina Ruzicska), the natural cleavage site is replaced by the Lb^{pro} self-processing sequence (QRKLK↓GAGQS). Both fragments were used in processing assays with either Lb^{pro} wt or Lb^{pro} L143A.

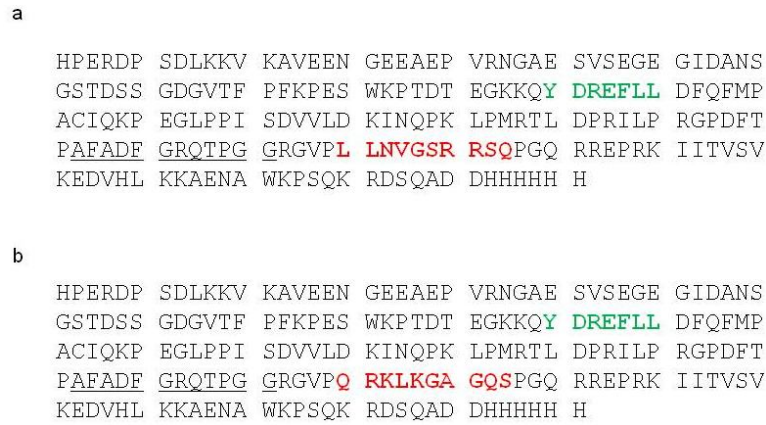


Figure 24: Amino acid sequences of the human eIF4GII fragment and eIF4GII^{self}. (a) eIF4GII fragment (aa 552-745) containing the wt cleavage sequence LLNVG↓SRRSQ recognized by Lb^{pro} wt. (b) eIF4GII^{self} fragment (aa 552-745). The natural cleavage site is replaced by the Lb^{pro} self-processing sequence QRKLK↓GAGQS. The eIF4E binding sequence is shown in green (Gradi *et al.* 2004) and the related sequence not recognized is underlined (Kuehnel *et al.* 2004). Accession number of full-length human eIF4GII: AF012072.

The expression of the eIF4GII fragment was performed in LB Amp/Cam medium using *E. coli* BL21(DE3)pLysS cells (3.3.1). After induction with 0.1 mM IPTG at an OD₆₀₀ between 0.5 - 0.6, protein expression was continued for 5 h at 30°C. The 25 kDa eIF4GII fragment could be observed directly after sonication (figure 25). Most of it is found in the soluble fraction.

eIF4GII is tagged with six histidine residues. Therefore, affinity chromatography was performed using a 1 ml HisTrap HP column (GE Healthcare) (3.3.4). The expressed eIF4GII (40 ml) from 4 liters of culture was loaded and separated using a one step gradient of HisTrap elution buffer. The chromatogram is shown in figure 26a (1st run/chromatography). The fractions A9-B6 were analyzed on the 17.5% SDS gels

presented in figure 26b. Fractions containing eIF4GII, A9-B6 (each 1 ml), were pooled and concentrated up to 500 µl for subsequent size-exclusion chromatography.

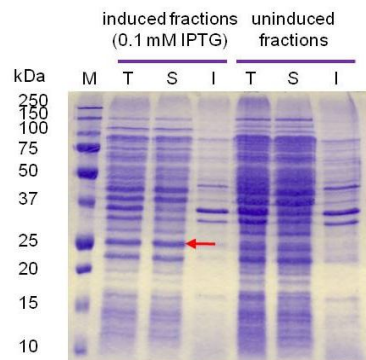


Figure 25: Total (T), soluble (S) and insoluble (I) fractions of the eIF4GII-fragment. 1 µl of the induced fractions (total of 40 ml) from expression in 4 L of culture was loaded on the 17.5% SDS gel and 9 µl of the uninduced fractions. The red arrow indicates the eIF4GII fragment running at about 25 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

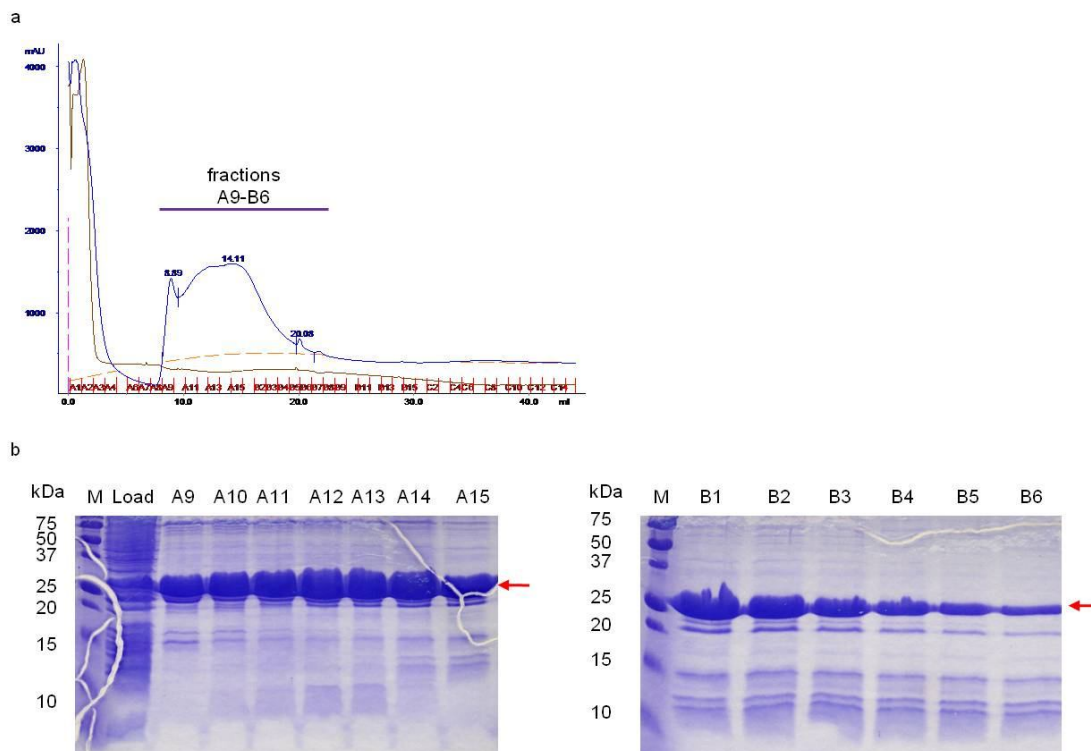


Figure 26: Purification of eIF4GII by affinity chromatography using a 1ml HisTrap HP column (1st run/chromatography). (a) Chromatogram of eIF4GII purified by affinity chromatography using a 1 ml HisTrap HP column. 40 ml of the eIF4GII sample expressed in 4 L of culture were loaded. The brown line represents the conductivity. Fractions analyzed by SDS PAGE are indicated. (b) 17.5% SDS gels of fractions collected during 1st run/chromatography. 2 µl of each fraction (total of 1 ml) and 1 µl of the loaded sample (Load) are present on the gel. Fractions A9-B6 were pooled and concentrated up to 500 µl. The red arrow indicates the eIF4GII fragment running at about 25 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

The flow-through (39 ml) collected during the loading for the 1st run/chromatography (figure 26a) was loaded again on a 1 ml HisTrap HP column (2nd run/chromatography). The chromatogram of the second run is presented in figure 27a. Fractions A9-B6 were examined on the 17.5% SDS gel shown in figure 27b. Again, fractions A9-B6 were pooled and concentrated up to 500 µl for further size-exclusion chromatography.

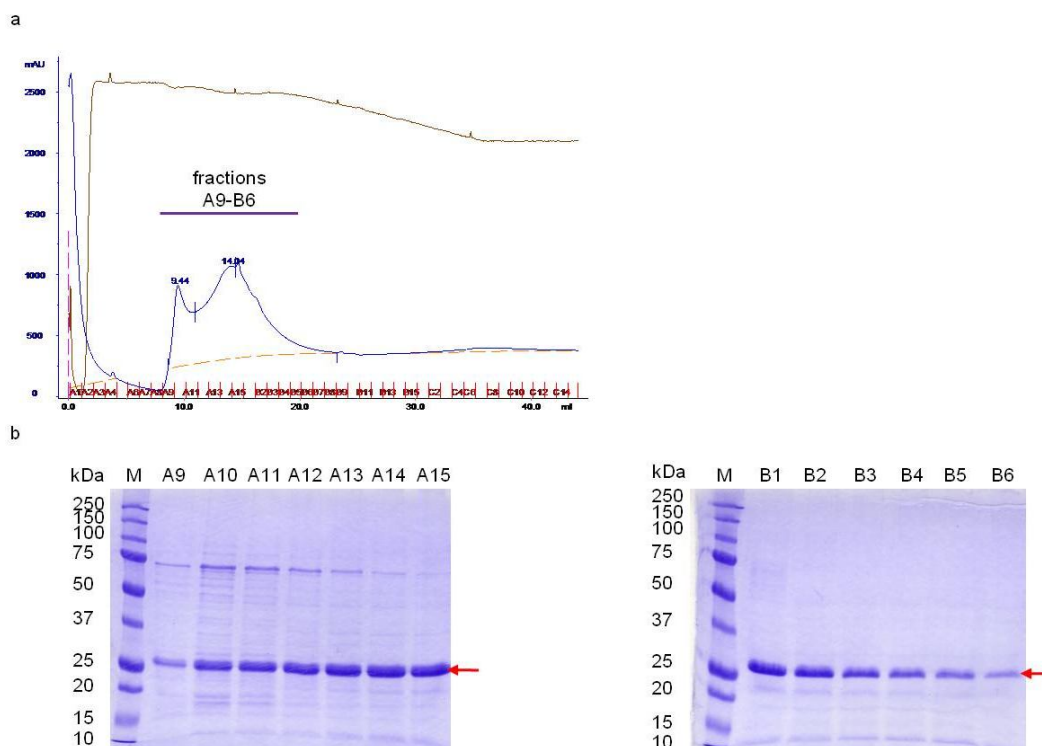


Figure 27: Purification of eIF4GII by affinity chromatography using a 1ml HisTrap HP column. (2nd run /chromatography) (a) Chromatogram of the 2nd run/chromatography loading the flow-through collected during the 1st run/chromatography (39 ml). Fractions analyzed by SDS PAGE are indicated. (b) 17.5% SDS gels of fractions collected during the 2nd run /chromatography. 2 µl of each 1 ml fraction are present on the gel. Fractions containing eIF4GII, A9-B6, were pooled and concentrated up to 500 µl for size-exclusion. The red arrow indicates the eIF4GII fragment running at about 25 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

The concentrated fractions of both runs (1 ml) using 1 ml HisTrap HP column were pooled and purified by size-exclusion chromatography using a 26/60 HiLoad Superdex 75 pg column (3.3.4). The chromatogram of the separation by size-exclusion chromatography is shown in figure 28a. Fractions D8-E15, the loaded sample as well as the flow-through were examined on 17.5% SDS gels shown in figure 28b. Fractions D10-E3 and E8-E15 were pooled and concentrated up to 1 ml. The fractions E4-E7 were not as pure as the other fractions and were therefore not used for further experiments.

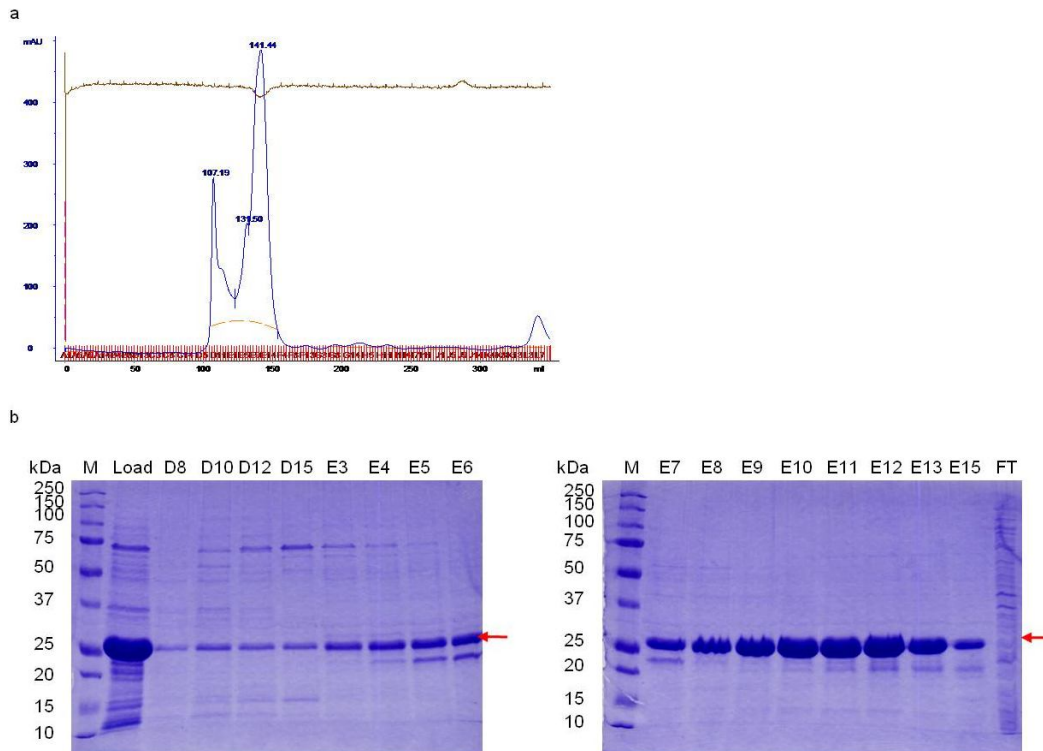


Figure 28: Purification of eIF4GII by size-exclusion chromatography using a 26/60 HiLoad Superdex 75 pg column. (a) Chromatogram of eIF4GII purified by size-exclusion chromatography. 1 ml of the eIF4GII purified by affinity chromatography was loaded. The brown line represents the conductivity. (b) 17.5% SDS gels of fractions collected during size-exclusion. 9 μ l of each fraction (total of 2 ml) and 1 μ l of the loaded sample (Load) and 1 μ l of the flow-through (FT) are present on the gel. Fractions D10-E3 were pooled and concentrated up to 1 ml. The red arrow indicates the eIF4GII fragment running at about 25 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

The second fragment eIF4GII^{self} contains the Lb^{pro} self-processing site instead of the natural cleavage site. The purification procedure was similar. eIF4GII^{self} was expressed for 5 h at 30°C in 2L of LB Amp/Cam culture (3.3.1). The total, soluble and insoluble fractions obtained after sonication are shown in figure 39.

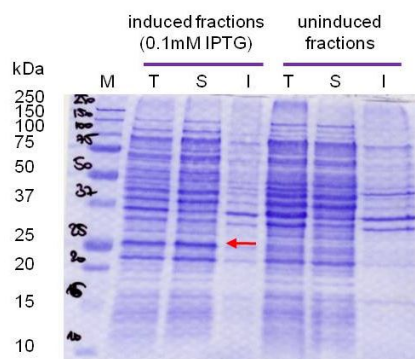


Figure 39: Total (T), soluble (S) and insoluble (I) fractions of the eIF4GII^{self} fragment. 1 μ l of the induced fractions (total of 20 ml for eIF4GII^{self}-from expression in 2 L of culture) was loaded on the 17.5% SDS gel and 9 μ l of the uninduced fractions. The red arrow indicates the eIF4GII^{self} fragment running at about 25 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

Subsequently, affinity chromatography was performed as the eIF4GII^{self} fragment is as well histidine tagged (3.3.4). A one step gradient of HisTrap elution buffer was used for the separation. The 20 ml of the eIF4GII^{self} sample obtained after sonication were divided and only 10 ml put on a 1 ml HisTrap HP column at once, as large amounts of protein failed to bind in previous experiments. Therefore, two runs were needed. The chromatograms of the 1st and 2nd run are shown in figure 30a and b, respectively.

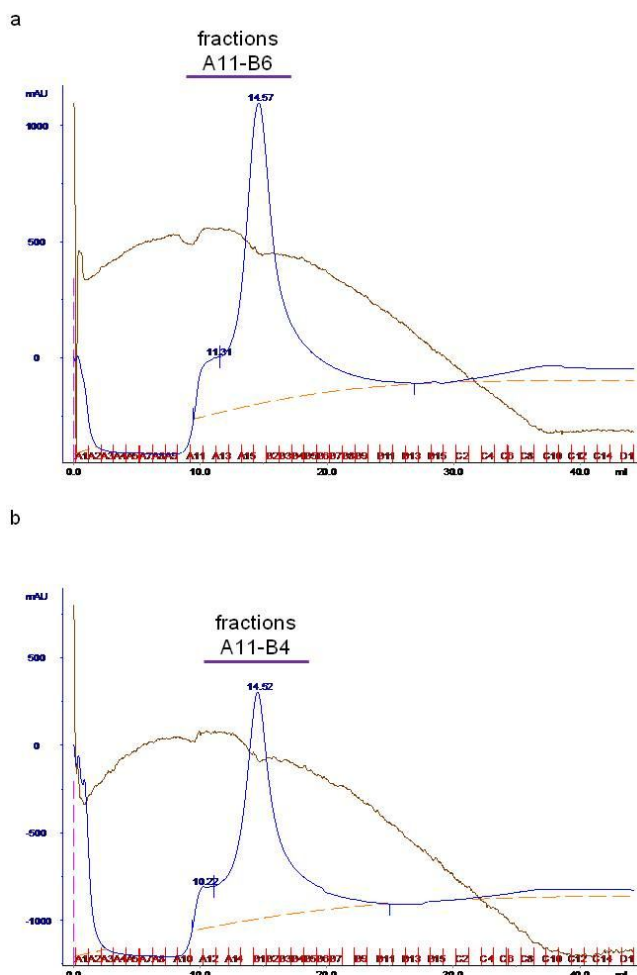


Figure 30: Purification of eIF4GII^{self} by affinity chromatography using a 1ml HisTrap HP column. (a) Chromatogram of the 1st run/chromatography loading 10 ml of the eIF4GII^{self} sample from an expression in 2 L of culture. (b) Chromatogram of the 2nd run/chromatography loading the second 10 ml of the 2 L eIF4GII^{self} expression. The brown line represents the conductivity. Fractions analyzed by SDS PAGE are indicated.

Fractions A11-B4 (1ml each) of the 1st run/chromatography were analyzed by 17.5% SDS gel (figure 31a). Fractions, A11-B4, of the 2nd run/chromatography were also examined by SDS PAGE shown in figure 31b. Fractions A15-B4 of both runs were pooled and concentrated up to 600 μ l for subsequent size-exclusion chromatography. Fractions A11-A14 of both runs were pooled and concentrated as well (600 μ l) and stored at -80 $^{\circ}$ C.

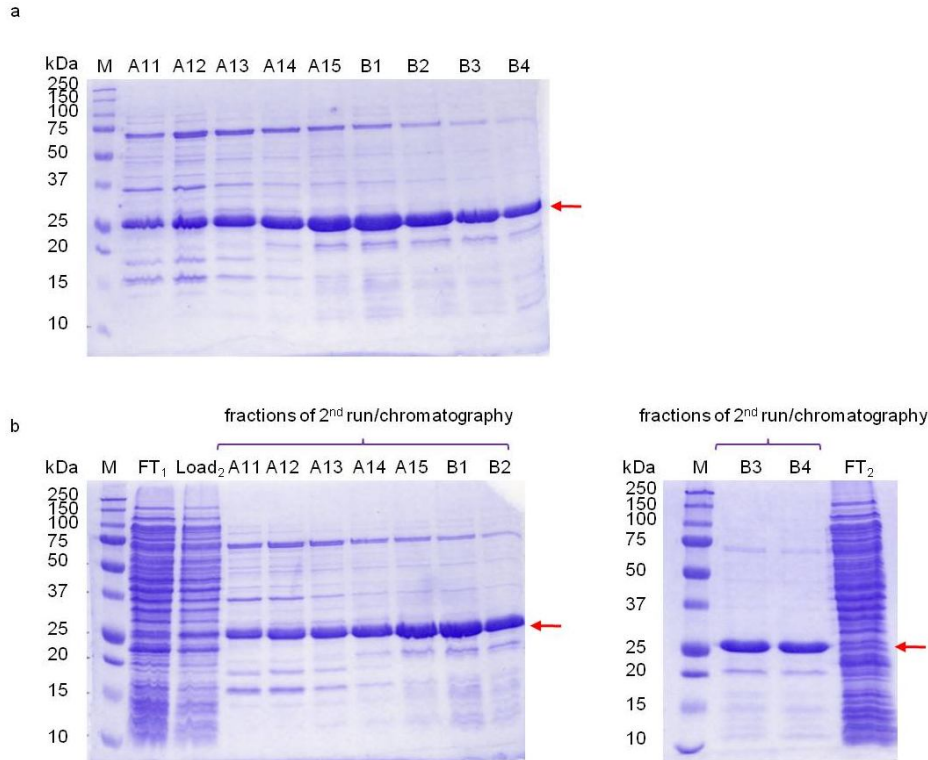
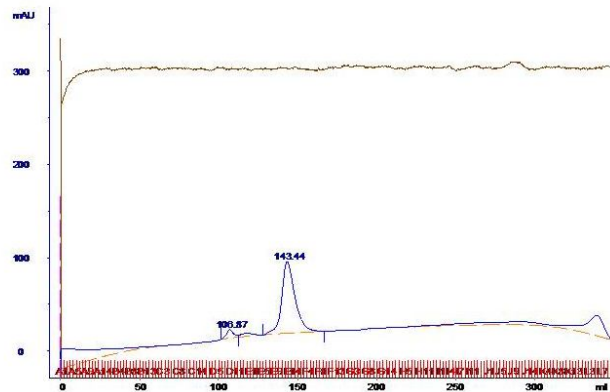


Figure 31: 17.5% SDS gels of fractions collected during eIF4GII^{self} purification using a 1ml HisTrap HP column. eIF4GII^{self} was expressed in 2 L of culture. **(a)** 9 μ l of the 1ml fractions A11-B4 of the 1st run are present on the gel. **(b)** 9 μ l of the flow-through collected during the 1st run (FT₁) and 2nd run (FT₂), 9 μ l of the sample loaded (Load₂) for the 2nd run and 9 μ l of A11-B4 (total of 1 ml) of the 2nd run are present on the gel. Fractions A15-B4 of both runs were pooled and concentrated up to 600 μ l. The red arrow indicates the eIF4GII^{self} fragment running at about 25 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

Following affinity chromatography, the pooled and concentrated eIF4GII^{self} sample was loaded on a 26/60 HiLoad Superdex 75 pg column. The pooled fractions A15-B4 of both runs were loaded (1.2 ml). The chromatogram is presented in figure 32a. The 2 ml fractions E10-E15 and the loaded sample were examined by SDS PAGE (figure 32b). Fractions containing eIF4GII^{self}, E10-E15, were pooled and concentrated up to 600 μ l for further processing experiments.

a



b

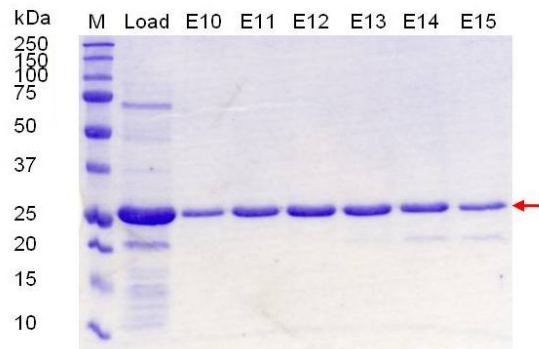


Figure 32: Purification of eIF4GII^{self} by size-exclusion chromatography using a 26/60 HiLoad Superdex 75 pg column. (a) Chromatogram of eIF4GII^{self} purified by size exclusion chromatography. 600 μ l of eIF4GII^{self} purified by affinity chromatography was loaded. The brown line represents the conductivity. (b) 17.5% SDS gel of fractions collected during size-exclusion. 9 μ l of each fraction (total of 2 ml) and 1 μ l of the loaded sample (Load) are present on the gel. Fractions E10-E15 were pooled and concentrated up to 600 μ l. The red arrow indicates the eIF4GII^{self} fragment running at about 25 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

The purified proteins, eIF4GII and eIF4GII^{self}, were examined by SDS PAGE (figure 33). The concentration of the eIF4GII fragment was slightly higher (10 µg/µl) than the concentration of eIF4GII^{self} (2 µg/µl).

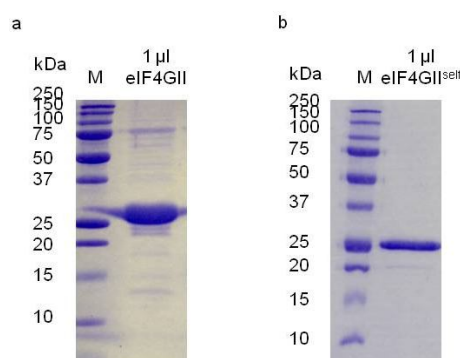


Figure 33: Purified (a) eIF4GII and (b) eIF4GII^{self}. 10 µg of eIF4GII and 2 µg of eIF4GII^{self} are present on the gel. The indicated amount was loaded on a 17.5% SDS gel. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

4.2.3 eIF4E

In contrast to the previous expressions, eIF4E was expressed in TB medium using *E. coli* BL21(DE3)pLysS cells (3.3.1). TB was used for expression due to its higher nutritional value which is needed to express satisfying amounts of eIF4E. 0.1 mM IPTG was used to induce the expression between an OD₆₀₀ of 0.5-0.6 for 5 h at 16°C. Figure 34 shows the total, soluble and insoluble fraction of eIF4E after sonication.

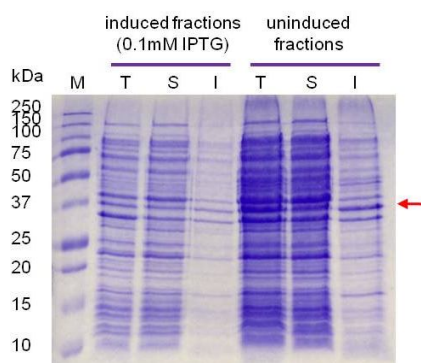


Figure 34: Total (T), soluble (S) and insoluble (I) fractions of eIF4E. 1 µl of the induced fractions (total of 40 ml eIF4E from expression in 4 L of culture) was loaded on the 17.5% SDS gel and 9 µl of the uninduced fractions. eIF4E sample should be running at about 37 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

Even though, the eIF4E posses a histidine-tag, it has been demonstrated that purification by affinity chromatography is not successful. Instead, it is better to perform the separation by anion exchange chromatography with a MonoQ 10/100 GL column directly after sonication using a three step-gradient of imidazole (3.3.4). 40 ml of eIF4E (expression in 4L of medium) were loaded via a 50 ml super-loop onto the column. The chromatogram is shown in figure 35a. The flow-through collected during the 1st run was again loaded (figure 35b). Both chromatograms show that the separation by MonoQ 10/100 GL is not optimal and gives rise to a multitude of peaks.

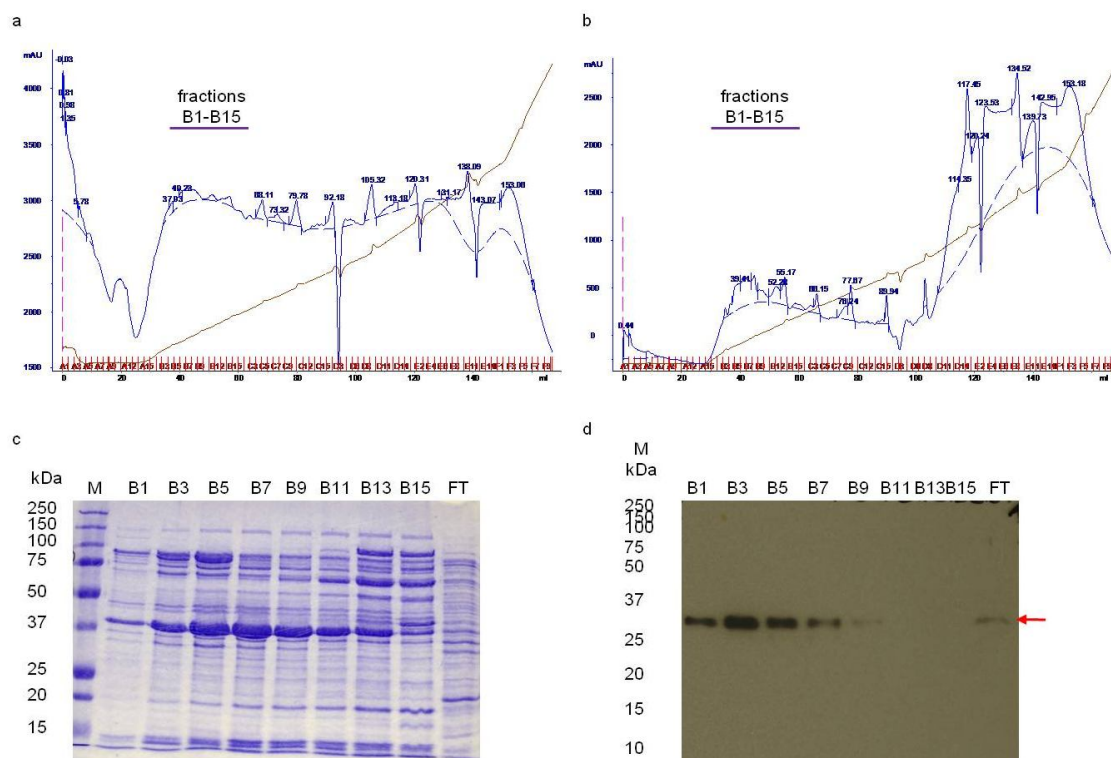


Figure 35: Purification of eIF4E by anion-exchange chromatography using a MonoQ 10/100 GL column. (a) Chromatogram of eIF4E purified by anion-exchange chromatography. 40 ml of the eIF4E expressed in 4L of culture were loaded. (1st run/chromatography). (b) The flow-through (40 ml) collected during the 1st run was loaded again (2nd run/chromatography). The brown line represents the conductivity. Fractions analyzed by SDS PAGE are indicated (c) 17.5% SDS gel of fractions collected during the 2nd run/ chromatography. 9 μ l of each fraction (total of 2 ml) and 1 μ l of the collected flow-through (FT) are present on the gel. Fractions B1-B9 were pooled and concentrated up to 500 μ l prior to separation by affinity chromatography using 1 ml 7-Methyl-GTP Sepharose 4B beads. (d) The same fractions as on the SDS PAGE were analyzed by western blot. 9 μ l of the 2 ml fractions and 1 μ l of the flow-through (FT) were loaded. The red arrow points at eIF4E running at about 35 kDa. An anti-His-antibody (Pierce) was used for detection. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

Thus, the first separation by anion-exchange chromatography yielded little eIF4E (data not shown) Therefore, only fractions of the 2nd run possibly containing eIF4E, B1-B3 (2ml fractions), were analyzed by SDS PAGE and western blot (figure 35c and d). A correct identification of the fractions containing eIF4E was only obtained by western blot analysis (3.3.9).

eIF4E is known to bind cellular mRNAs by interacting with the m⁷ GTP cap structure. This specific interaction was utilized for the last purification step by affinity chromatography using 1 ml of 7-Methyl-GTP Sepharose 4B beads that specifically bind eIF4E (3.3.4). After dialysis against the eIF4E binding buffer, the beads were incubated at 4°C overnight with 500 µl of concentrated sample. After the unbound sample (U1-U4) was washed out with eIF4E binding buffer (2 ml fractions), the beads were incubated for 3 h with 600 µl of eIF4E elution buffer. Bound eIF4E was eluted with 10 ml of eIF4E elution buffer and 1 ml fractions collected (E1-E13). Before regeneration, the beads were washed with 5 ml of eIF4E elution buffer and 2 ml fractions collected (W1-W3). The beads were regenerated as described in 3.3.6.4. All fractions collected during the washing (U1-U4 and W1-W3) and elution steps (E1-E13) were analyzed by SDS PAGE (figure 36). Fractions, E1-E13, containing pure eIF4E were pooled and concentrated up to 600 µl. The unbound fractions U1-U4 still contained high amounts of protein and were therefore also pooled, concentrated up to 500 µl and purified again using 7-Methyl-GTP Sepharose 4B beads (data not shown).

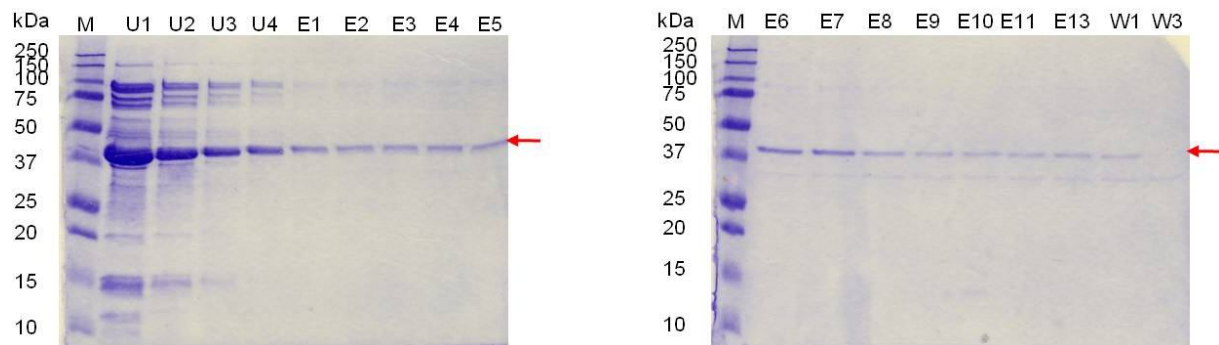


Figure 36: 17.5% SDS gels of eIF4E purification by affinity chromatography using 7-Methyl-GTP Sepharose 4B. 9 µl of the unbound fractions (U1-U4; total of 2 ml), 9 µl the eluted samples (E1-E13; total of 1 ml) and 9 µl of the wash fractions (W1-W3; total of 2 ml) were loaded. Fractions E1-E13 were pooled and concentrated up to 600 µl. U1-U4 were pooled and concentrated as well and incubated again with the beads. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

Before using eIF4E together with eIF4GII, eIF4GII^{self} and the leader protease, eIF4E was dialyzed against Buffer A. Figure 37 shows the purified eIF4E (pooled fractions E1-E13 as shown in figure 36) with a concentration of 0.5 µg/µl.

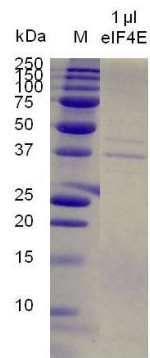


Figure 37: Purified eIF4E. 0.5 µg of eIF4E are present on the gel. The indicated amount was loaded on a 17.5% SDS gel. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

4.3 *In vitro* activity test with HeLa cell extract

To test whether the expressed and purified proteases are functional, an activity assay with HeLa cell extract was performed. HeLa cell extracts contain both isoforms of eIF4G which are cleaved by active leader proteases (Lloyd *et al.* 2006). Therefore, different amounts of protease (2 µl) were incubated with 8 µl of cell extract at 30°C for 60 min. Reactions were stopped by adding 5x sample buffer (3.3.11). The samples were separated by 6% SDS PAGE and analyzed by western blot using the alkaline phosphatase system for detection (3.3.9). Lb^{pro} performs a single cleavage thus separating the N-terminal one-third of eIF4G from the C-terminal two-thirds. Even though eIF4G has a predicted molecular mass of about 156 kDa, both homologues migrate as a series of bands on SDS PAGE, the lowest at 220 kDa. The reason for this heterologous mobility is a complex translation initiation scheme involving five different AUG initiation codons and alternative splicing producing five isoforms of eIF4G varying at the N-terminus (Lloyd *et al.* 2006).

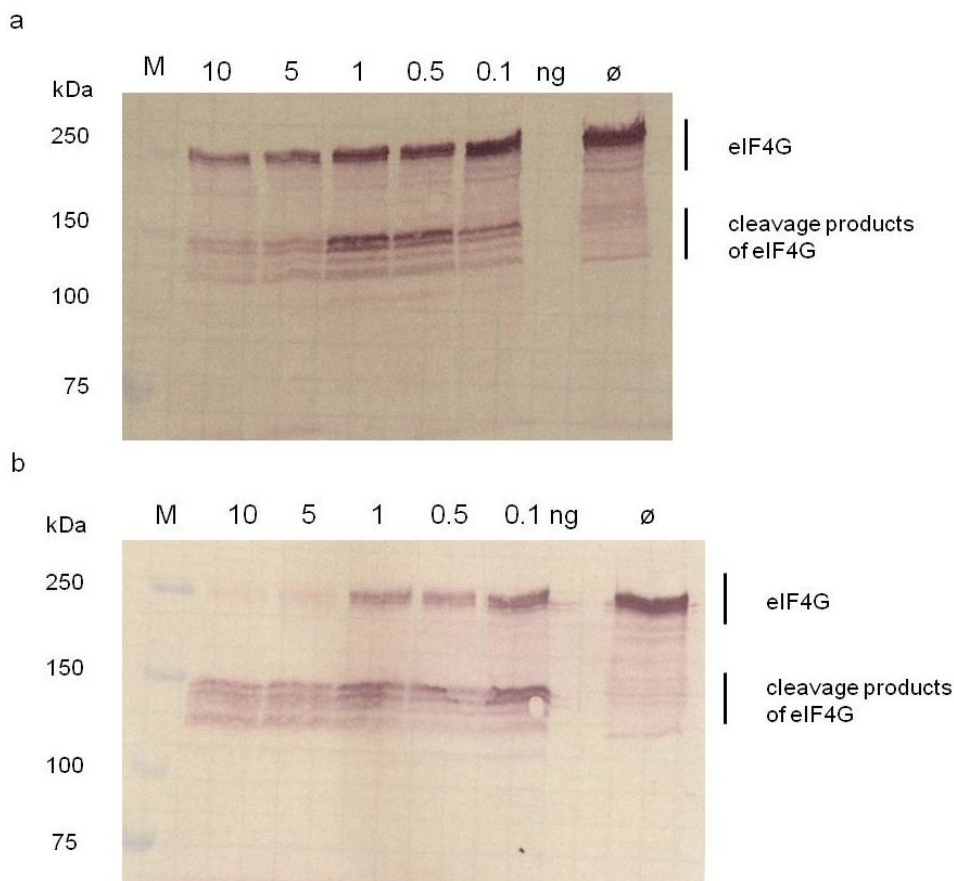


Figure 38: Western Blot of activity test with HeLa cell extract. (a) HeLa cell extract incubated with different amounts of Lb^{pro} L143A and (b) of Lb^{pro} wt. Detection with rabbit anti-eIF4G antibody and anti rabbit IgG alkaline phosphatase coupled (Sigma). All of the activity-test (10 µl) was loaded on the 6% SDS gel. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

The blot in figure 38b shows a good correlation between the amount of Lb^{pro} wt and the cleavage rate of eIF4G. In contrast, the cleavage with Lb^{pro} L143A (figure 38a) is not as consistent with the amount of protease used. As this was the first assay performed with both proteases, the concentrations were not the same for Lb^{pro} wt and Lb^{pro} L143A. Before further assays were performed, the concentrations were controlled by SDS PAGE (data not shown). Nevertheless, both proteases are active and able to cleave eIF4G. As expected Lb^{pro} wt cleaves more efficiently compared to Lb^{pro} L143A.

4.4 Processing assays

To investigate the different cleavage properties of Lb^{pro} wt and Lb^{pro} L143A, a variety of processing assays was performed and analyzed on 17.5% SDS gels. All of them contain either eIF4GII or the eIF4GII^{self} as substrate. In some cases eIF4E was added in different amounts. As described in 1.6.1, eIF4E binds the cap structure of cellular mRNAs and a particular domain of eIF4GII. This interaction is thought to further promote the cleavage of eIF4GII by proteases, as some experiments suggest that eIF4GII becomes more structured upon binding to eIF4E (Ohlmann *et al.* 1997). The hypothesis is that due to the the unfolded-to-folded transition, the Lb^{pro} cleavage site becomes exposed, thus being better accessible for Lb^{pro} and enhancing the processing event. We tried to reproduce these assumptions using Lb^{pro} wt and Lb^{pro} L143A. Furthermore, these assays should shed more light on the specificity of Lb^{pro} wt, as Leu143 was shown to be highly involved its maintenance (Mayer *et al.* 2008). The second substrate tested, eIF4GII^{self}, carries the self-processing site of Lb^{pro} QRKLK↓GAGQS instead of its wild-type cleavage sequence LLNVG↓SRRSQ. We wanted to test whether this site is processed even though the chemical environment is different.

4.4.1 Processing of eIF4GII with Lb^{pro} wt

The first cleavage performed with purified eIF4GII was with 0-20 ng of Lb^{pro} wt incubated for 60 min at 30°C (3.3.12). The leader protease cleaves the eIF4GII fragment into a smaller fragment of about 11 kDa (cp_C) and a larger one running at about 18 kDa (cp_N) (figure 39).

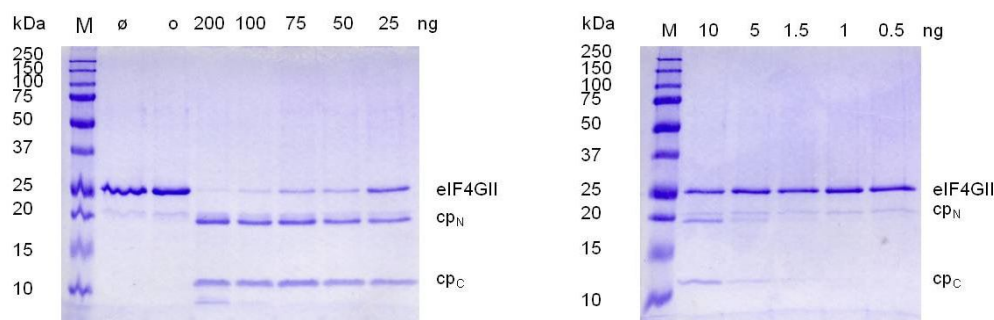


Figure 39: Processing assay with eIF4GII and Lb^{pro}. The amounts of protease indicated were incubated with 1 µg of eIF4GII for 60 min at 30°C. 1 µl of protease (in different amounts) was used together with 1 µl (= 1 µg) of eIF4GII and filled up with Buffer A to 13 µl. 13 µl of the assays and 12 µl of the negative controls (ø = 1 µg of eIF4GII not incubated; o = 1 µg of eIF4GII incubated) were loaded on a 17.5% gel. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

The smaller product contains the histidine tag after cleavage (see figure 24a). 50 ng of Lb^{pro} wt are sufficient for the cleavage of 50% eIF4GII after 60 min at 30°C. An additional band is observable in processing of eIF4GII with 200 ng of Lb^{pro} wt. However, this is most likely the front of the gel as a similar band is visible in the lane of the marker.

In subsequent experiments, a time course was performed to determine the point when 50% eIF4GII are processed by Lb^{pro} wt (figure 40). The incubation of 50 ng Lb^{pro} wt from 10-60min with eIF4GII shows no major differences regarding cleavage progress. Therefore, another time course from 2-10min was performed. In both cases, 50% of eIF4GII were cleaved with 50 ng Lb^{pro} wt after 10 min at 30°C.

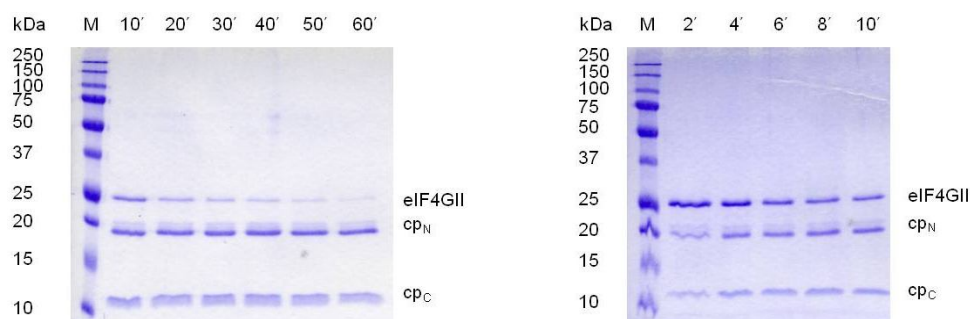


Figure 40: Time course of the processing assay with 50 ng Lb^{pro} wt and eIF4GII. 50 ng of Lb^{pro} wt were incubated for the indicated time (min) with 1 µg eIF4GII at 30°C. 13 µl were loaded on a 17.5% gel (2 µl of protease (total of 50ng) and 1 µl (= 1 µg) of eIF4GII filled up to 13 µl with Buffer A). cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

A recurring problem with both proteases was that after several assays the larger cleavage fragment (cp_N) was no longer visible as shown in figure 41a. Different approaches to restore this cleavage product were tested, but still the cause remains unclear. Neither the use of a new aliquot of eIF4GII (kept at -80°C) nor a new aliquot of Lb^{pro} wt were able to restore the larger cleavage product. Additionally, eIF4E was added to the processing assay, but the intensity of the larger cleavage product also did not rise (figure 41b). Moreover, we found out that the eIF4GII fragment precipitates during the assay (figure 41c) which might be associated with the problems concerning the larger cleavage product of eIF4GII.

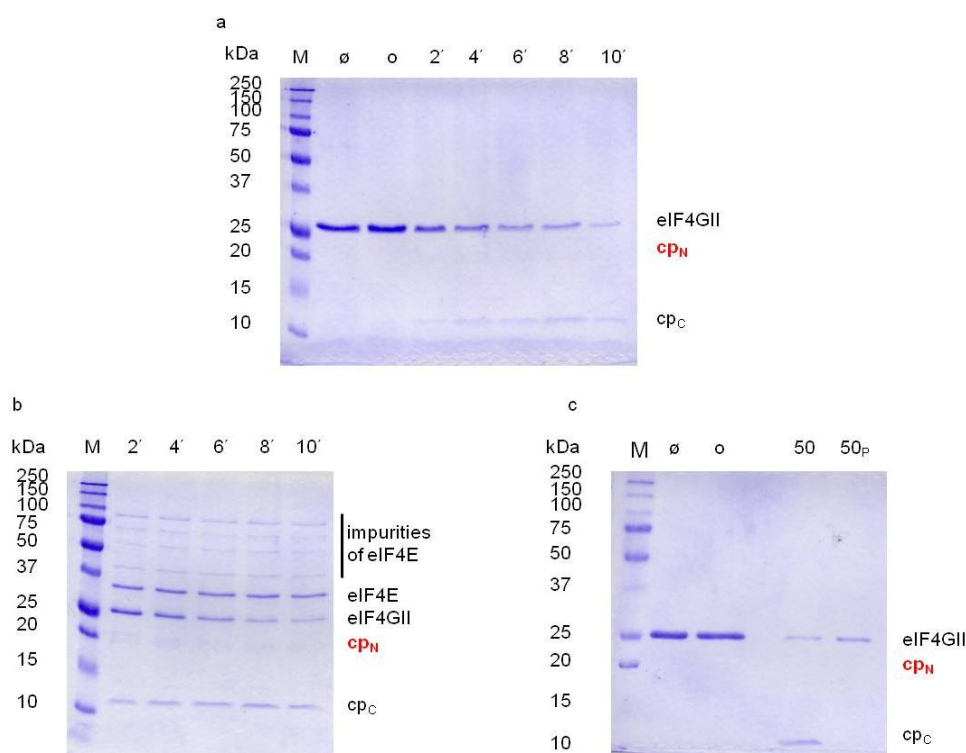


Figure 41: Processing assays of eIF4GII with 50 ng Lb^{pro} wt. (a) 1 μg of eIF4GII incubated for indicated time (min) with 50 ng of Lb^{pro} wt at 30°C . (b) Addition of 1 μg eIF4E to 1 μg eIF4GII prior incubation with 50 ng of Lb^{pro} wt at 30°C for the indicated time (min). (c) 50 ng of Lb^{pro} wt incubated with 1 μg eIF4GII for 10 min at 25°C . 50_p demonstrates the precipitated eIF4GII remaining in the tube after the incubated aliquot was taken out. $\emptyset$ represents the negative control of eIF4GII not incubated and o for eIF4GII incubated for 10 min at 30°C or 25°C respectively. eIF4GII and eIF4E are indicated, as is the smaller cleavage product (cp_C). The size of larger cleavage product is indicated by cp_N . M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μl loaded).

Due to these problems, the native state of the protease and the substrate were observed by blue native gel electrophoresis (BN PAGE) (3.3.10). This might shed more light on the reason of the hardly observable larger cleavage product as it is present in the native state. eIF4GII and the processing products should be visible on the BN PAGE if they are in their native and folded state.

However, the larger cleavage product reappeared in subsequent processing assays again.

4.4.2 Processing of eIF4GII with Lb^{pro} L143A

The procedure for the processing with Lb^{pro} L143A remains the same as with Lb^{pro} wt (3.3.12), as the parameters were important to compare the cleavage ability of the two constructs. The precipitation of eIF4GII occurs also during the incubation with Lb^{pro} L143A. For each amount of Lb^{pro} L143A a new tube was used. After 10 min the entire sample was taken out, the reaction stopped with 5x sample buffer and separated by SDS PAGE (figure 42). Buffer A was added to the empty tube and analyzed by SDS PAGE (50_P, 25_P, 10_P and 5_P). About half of the amount of eIF4GII (0.5 µg) were observed to precipitate during the assay. Besides the precipitation, the aberrant processing products generated by cleavage with Lb^{pro} L143A were also visible as additional bands around cp_N.

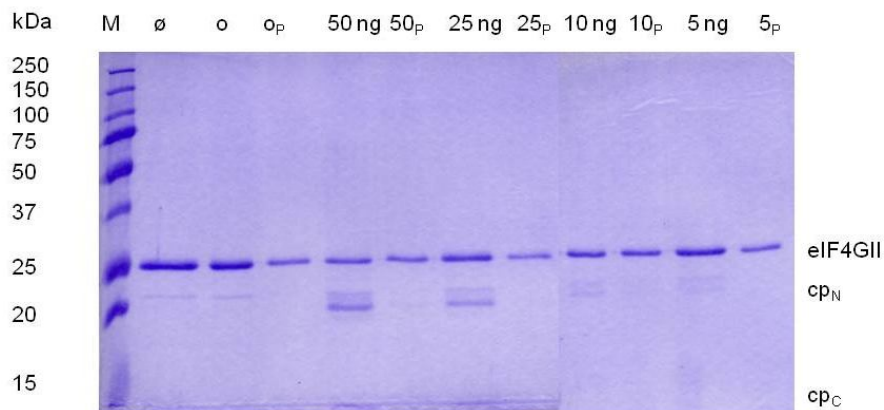


Figure 42: Processing assay with different amounts of Lb^{pro} L143A and eIF4GII. The amounts indicated on top were incubated with 1 µg of eIF4GII for 10 min at 25 °C. For each amount a separate tube was taken and the precipitant (50_P, 25_P, etc.) remaining after taking all of the sample out analyzed on a 17.5% SDS gel. ø, o and o_P indicate the negative controls not incubated, incubated and the precipitant left in the incubated tube, respectively. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

A time course with eIF4GII and 50 ng of Lb^{pro} L143A was performed as well. The point of 50% eIF4GII cleavage was determined to be 50 ng of Lb^{pro} L143A incubated for 10 min at 30°C (figure 43).

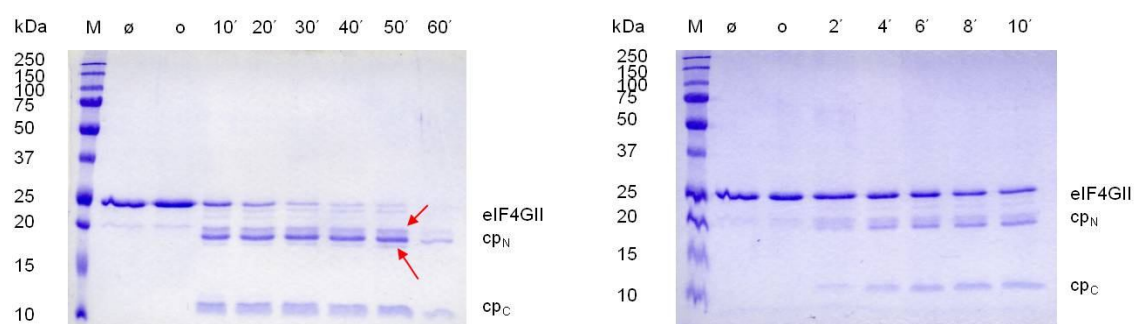


Figure 43: Time course of the processing assay with 50 ng Lb^{pro} L143A and eIF4GII. 50 ng of Lb^{pro} L143A were incubated for the indicated time (min) with 1 µg eIF4GII at 30°C. 13 µl were loaded on a 17.5% gel (2 µl of protease (total of 50ng) and 1 µl (= 1 µg) of eIF4GII filled up to 13 µl with Buffer A). cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. The red arrows point at the aberrant cleavage products generated only with Lb^{pro} L143A not with Lb^{pro} wt. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

The processing of eIF4GII with Lb^{pro} wt produces only two cleavage products, cp_N and cp_C (figure 39 and 40). In contrast, the mutant form Lb^{pro} L143A gives rise to some additional cleavage products besides cp_N and cp_C (figure 43 and 44). Mayer *et al.* proposed that the specificity of the Lb^{pro} L143A is lower than the one of the wild-type protease (Mayer *et al.* 2008). Therefore, Lb^{pro} L143A is expected to recognize additional sites thus producing additional cleavage products. As these aberrant products could provide information about alternative sequences recognized by Lb^{pro} L143A, two additional cleavage products were analyzed by mass spectrometry (3.3.13).

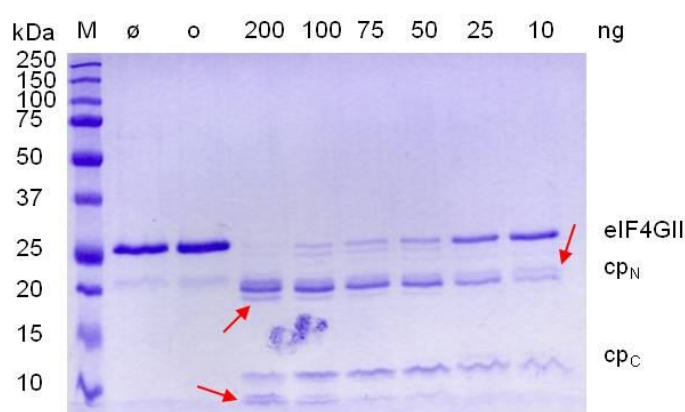


Figure 44: Processing assay of eIF4GII with Lb^{pro} L143A. The amounts indicated on top were incubated with 1 µg of eIF4GII for 60 min at 30°C and separated on a 17.5% SDS gel. ø and o represent the not incubated and incubated negative control, respectively. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. The red arrows point at the aberrant cleavage products generated only with Lb^{pro} L143A not with Lb^{pro} wt. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

4.4.1 Mass spectrometry on eIF4GII cleavage products generated by Lb^{pro} L143A

Lb^{pro} L143A generates more cleavage products on eIF4GII than Lb^{pro} wt. Two of these additional processing products were chosen and analyzed using mass spectrometry (3.3.13). The processing assays were performed with 1 µl of eIF4GII and either 100 ng or 50 ng of Lb^{pro} wt or Lb^{pro} L143A. The reaction was stopped with sample buffer after 40 or 50 min of incubation at 30°C and the samples were separated on a 17.5% SDS gel. In contrast to former processing assays, more attention to the handling was given. Especially any contamination with dust particles, hair or skin should be prevented. Therefore, all solutions were filtered through 0.22 µm sterile membrane filters and the whole equipment properly washed with ethanol. Following the electrophoresis, the gel was stained with Coomassie and destained with a destaining solution (30% methanol, 8% acetic acid) in a closed dish. The gel bands highlighted in figure 45 were cut out under the lamina flow and covered with SDS running buffer. The two cleavage products were separately analyzed by mass spectrometry and the data compared to the nr-database and to a small internal database (containing often occurring contaminations as creatine, common proteins but also proteases used during the experiment). The results for both samples are shown in figure 46 and 47.

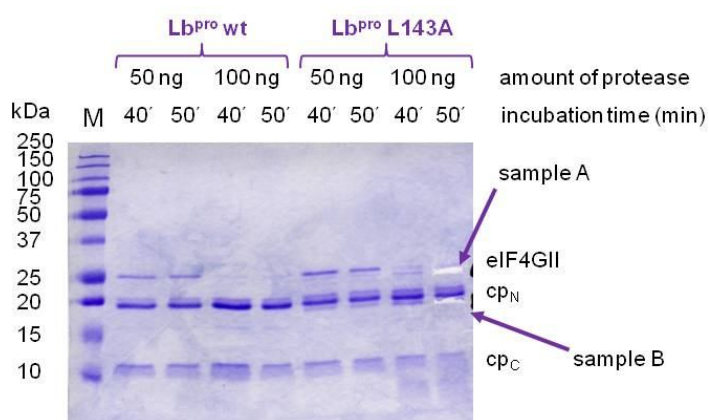


Figure 45: Processing assay of Lb^{pro} wt /Lb^{pro} L143 A with eIF4GII for mass spectrometry. 100 ng and 50 ng of both proteases were incubated for 40 and 50 min at 30°C before loading on a 17.5% SDS gel. The samples for mass spectrometry analysis were cut out. Sample A and B are indicated with arrows. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

The following parameters were used for the measurements of both samples:

Enzyme: Trypsin

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Mass values: Monoisotopic

Protein Mass: Unrestricted

Peptide Mass Tolerance: ± 5 ppm

Fragment Mass Tolerance: ± 0.8 Da

Max Missed Cleavages: 2

Instrument type: LTQ Orbitrap Velos

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1 HPERDPSDLK KVKAVEENG EAEPVRNGAE SVSEEGEGIEA NSGSTDSSGD
51 GVTFFPKPES WKPTDTEGKK QYDREFLLDF QFMPACIQKP EGLPPISDVV
101 LDKINQPKLP MRTLDPRILP RGPDFTPAFA DFGRQTPGGR GVPLLNVGSR
151 RSQPGQRREP RKIITVSVKE DVHLKKAENA WKPSQKRDSQ ADDHHHHHH
```

Figure 46: Matched peptides for sample A. In red the matching peptides are shown, resulting from the comparisons of sample A with the two databases.

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1 HPERDPSDLK KVKAVEENG EAEPVRNGAE SVSEEGEGIEA NSGSTDSSGD
51 GVTFFPKPES WKPTDTEGKK QYDREFLLDF QFMPACIQKP EGLPPISDVV
101 LDKINQPKLP MRTLDPRILP RGPDFTPAFA DFGRQTPGGR GVPLLNVGSR
151 RSQPGQRREP RKIITVSVKE DVHLKKAENA WKPSQKRDSQ ADDHHHHHH
```

Figure 47: Matched peptides for sample B. In red the matching peptides are highlighted, resulting from the comparisons of sample B with the two databases.

Several peptides corresponding to the eIF4GII fragment were found and matched to the sequence. However, contaminations of other proteins cannot be excluded. An additional cleavage site could not be determined by mass spectrometry analysis on sample A and B. The related sequence AFADFGX^RQTPGG (X stands for the potential site of cleavage) in eIF4GII was expected to be recognized by Lb^{pro} L143A. This was not confirmed by an explicit sequence received by mass spectrometry. Furthermore, no other potential cleavage sites were observed.

4.4.2 Processing of eIF4GII^{self} with Lb^{pro} wt

In addition to the eIF4GII fragment, a second fragment termed eIF4GII^{self} was used for processing assays. This fragment contains the self-processing site of the leader protease QRKLK↓GAGQS instead of its wild-type cleavage sequence LLNVG↓SRRSQ. The chemical environment of eIF4GII is totally different than the one present during the self-processing reaction.

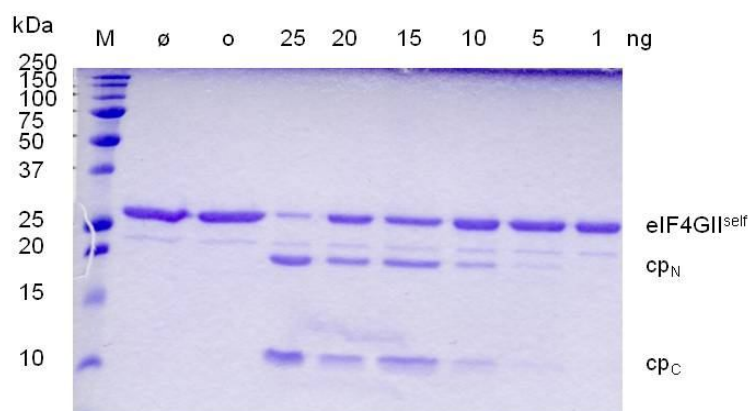


Figure 48: Processing assay of eIF4GII^{self} with different amounts of Lb^{pro} wt. 1 µg of eIF4GII^{self} was incubated with different amounts of Lb^{pro} wt for 10 min at 30°C. ø and o represent the negative controls which are either not incubated or incubated for 10 min, respectively. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII^{self}, respectively. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

However, the sequence was recognized and cleaved by Lb^{pro} wt. Figure 48 shows that 50% of eIF4GII^{self} were already cleaved with 15 ng of Lb^{pro} wt after 10 min at 30°C. In contrast, to obtain 50% of cleavage with eIF4GII fragment containing the wild-type cleavage site, 50 ng of the wild-type protease were needed.

4.4.3 Processing of eIF4GII^{self} with Lb^{pro} L143A

The cleavage properties of Lb^{pro} L143A were also tested with eIF4GII^{self}. Figure 49 reveals that 6.25 ng of Lb^{pro} L143A are sufficient for processing 50% of eIF4GII^{self}. The amount of protease needed to cleave eIF4GII^{self} is also much lower than for eIF4GII for which 50 ng of Lb^{pro} L143A are required.

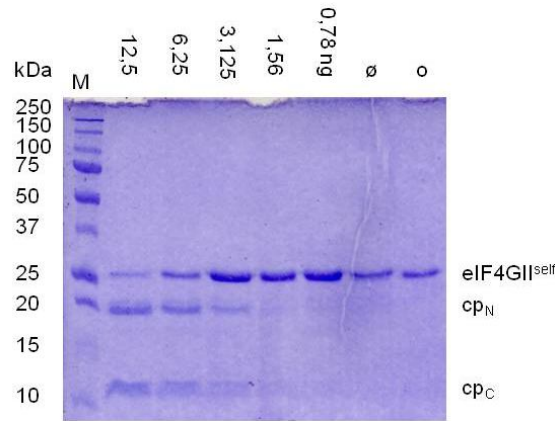


Figure 49: Processing assay of eIF4GII^{self} with different amounts of Lb^{pro} L143A. 1 µg of eIF4GII^{self} was incubated with different amounts of Lb^{pro} L143A for 10 min at 30°C. ø and o represent the negative controls which are either not incubated or incubated for 10 min, respectively. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII^{self}, respectively. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

4.4.4 Processing of eIF4GII with Lb^{pro} wt or Lb^{pro} L143A in presence of eIF4E

During cap-dependent translation initiation, eIF4GII works as scaffolding protein co-ordinating the components of the translation initiation complex eIF4F. Once eIF4GII binds the cap-binding protein eIF4E, it is thought to undergo an unfolded-to-folded transition. Hence, the cleavage site for Lb^{pro} on eIF4GII is thought to become more accessible to the protease, thus enhancing the processing event (Ohlmann *et al.* 1997, Gross *et al.* 2003). We wanted to reproduce these findings by incubating eIF4GII together with eIF4E overnight at 4°C to enable complex formation prior to cleavage (3.3.12). First, a 1:1 ratio of eIF4GII-eIF4E (1µg of each) was chosen and the complex was incubated with 50 ng of protease up to 60 min at 30°C. The incubation of the complex with 50 ng of Lb^{pro} wt is shown in figure 50a. Figure 50b shows the processing assay of eIF4GII-eIF4E with Lb^{pro} L143A. With both proteases, most changes occur within the first ten minutes of incubation as no major differences are observed when incubated longer. Furthermore, no significant improvement of cleavage can be observed when compared to the processing assays of Lb^{pro} wt or Lb^{pro} L143A with only eIF4GII (figure 40 and 43, respectively).

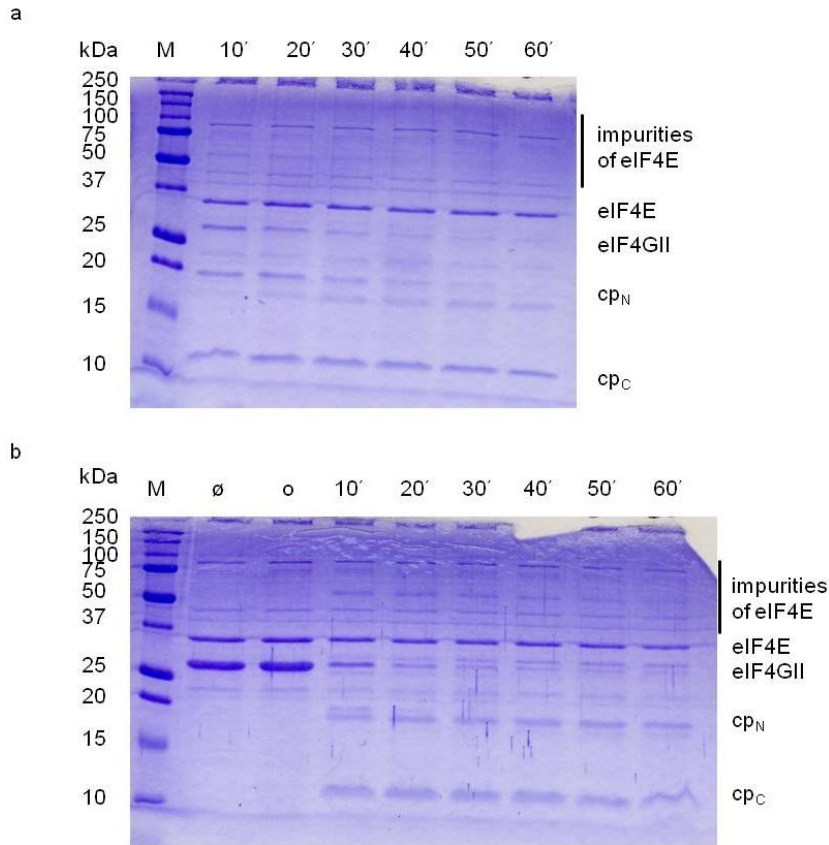


Figure 50: Time course of the processing assay of 1:1 ratio of eIF4GII:eIF4E with 50 ng (a) Lb^{pro} wt or (b) Lb^{pro} L143A. 50 ng of protease were incubated for the indicated time (min) with eIF4GII-eIF4E at 30°C. 13 μ l were loaded on a 17.5% gel (2 μ l of protease (50 ng) and 2 μ l of the eIF4GII-eIF4E complex (1 μ g each) filled up to 13 μ l with Buffer A). cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. Impurities of the eIF4E sample run between 100-40 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μ l loaded).

In addition, 1:1, 1:2 and 1:3 ratios of eIF4GII:eIF4E were examined (figure 51). No major differences were observed with higher ratios of eIF4GII:eIF4E. Before, the point of 50% eIF4GII processing was determined to 50 ng of Lb^{pro} wt or Lb^{pro} L143A after 10 min incubation at 30°C. Therefore, the processing assay with the eIF4GII-eIF4E complex was performed at 10 min and 30°C. However, the 50% cleavage point is not as clearly as before and slightly more eIF4GII seems to be cleaved in presence of eIF4E after 10 min. Furthermore, two larger processing products cp_N were obtained with Lb^{pro} L143A; a very faint one of the same size as cp_N (18 kDa) generated with Lb^{pro} wt, the second is slightly smaller (16 kDa) than the cp_N after cleavage with Lb^{pro} wt. The one running at about 16 kDa is more prominent than the one at 18 kDa.

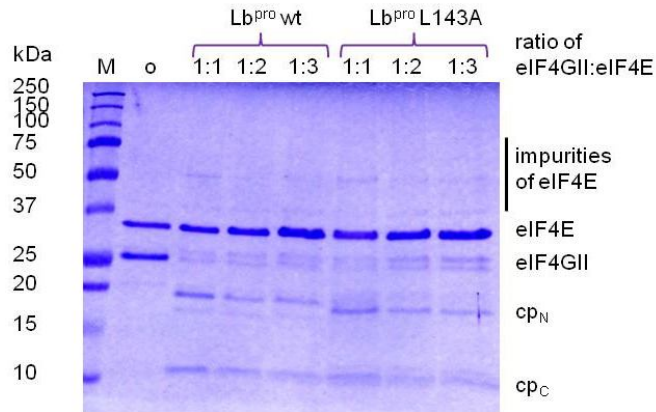


Figure 51: Processing assay with different ratios of eIF4GII:eIF4E and 50 ng Lb^{pro} wt or Lb^{pro} L143A. 50 ng of the protease were incubated with a 1:1, 1:2 or 1:3 ratio of eIF4GII:eIF4E for 10 min at 30°C. o represents the incubated 1:1 ratio of eIF4GII:eIF4E. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. Impurities of the eIF4E sample run between 100-40 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

According to Slepnev *et al.*, the optimal ratio of eIF4GII:eIF4E is with equal or lower amounts of eIF4E (Slepnev *et al.* 2008). Hence, 1:1, 1:0.5 and 1:0.25 ratios of eIF4GII:eIF4E were incubated prior to cleavage with either Lb^{pro} wt or Lb^{pro} L143A (figure 52). The size difference of cp_N obtained with Lb^{pro} L143A is again visible when incubated with equal and lower amounts of eIF4E. Besides this, no major differences were observed when different ratios of eIF4E were used compared to the processing assays using only eIF4GII.

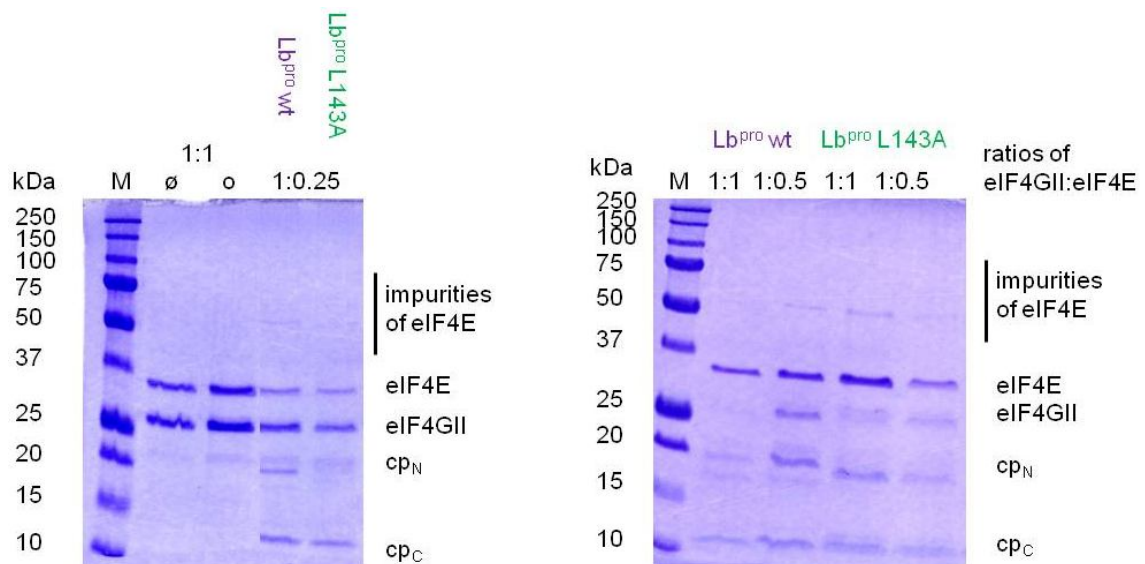


Figure 52: Processing assay with different ratios of eIF4GII:eIF4E and 50 ng Lb^{pro} wt or Lb^{pro} L143A. 50 ng of the protease were incubated with a 1:1, 1:0.5 or 1:0.25 ratio of eIF4GII:eIF4E for 10 min at 30°C. ø and o represents the not incubated and incubated 1:1 ratio of eIF4GII:eIF4E. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. Impurities of the eIF4E sample run between 100-40 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

4.4.5 Processing of eIF4GII^{self} with Lb^{pro} wt or Lb^{pro} L143A in presence of eIF4E

The assays described in 4.4.4 were performed similarly way the eIF4GII^{self} fragment. In contrast to eIF4GII, eIF4GII^{self} as substrate produced only cleavage products of the same size (18 kDa) with both proteases (figure 53). The ratio of eIF4GII^{self} remained unaltered, whereas the amount of eIF4E was changed. For the processing assays the amount of protease cleaving 50% of eIF4GII^{self} after 10 min at 30°C was chosen. These were 15 ng in case of Lb^{pro} wt and 6.25 ng of Lb^{pro} L143A. As with eIF4GII:eIF4E no significant differences were observed with the complex of eIF4GII^{self}:eIF4E. However, the size difference of cp_N observed with Lb^{pro} L143A cleaving eIF4GII in the binary complex was not detectable when eIF4GII^{self} was used as substrate with eIF4E.

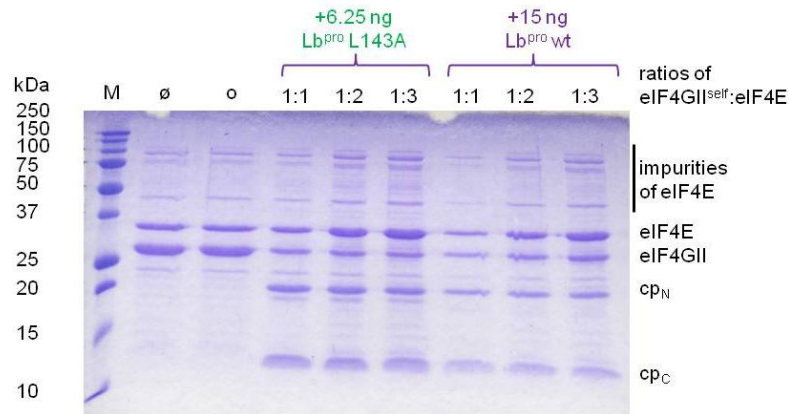


Figure 53: Processing assay with different ratios of eIF4GII^{self}:eIF4E and 15 ng Lb^{pro} wt or 6.25 ng Lb^{pro} L143A. The protease was incubated with a 1:1, 1:2 or 1:3 ratio of eIF4GII^{self}:eIF4E for 10 min at 30°C. ø and o represents the not incubated and incubated 1:1 ratio of eIF4GII^{self}:eIF4E. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII^{self}, respectively. Impurities of the eIF4E sample run between 100-40 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 µl loaded).

4.4.6 Processing of eIF4GII with Lb^{pro} wt in presence of eIF4E, m⁷GTP

As mentioned in 1.6.1, the cap binding protein eIF4E interacts with the 5' cap structure of host cell mRNAs. The structure present at the 5' end of the host mRNA is a 7-methylguanosine linked to the mRNA via a triphosphate bridge. As eIF4E binds to this structure, the use of additional m⁷GTP in the processing assays might enhance the binding of eIF4GII to eIF4E as proposed by Haghighat *et al.* 1997. Hence, the ternary complex of cap, eIF4E and eIF4GII might be a better substrate for the protease. 100 µM m⁷GTP were added to eIF4G:eIF4E in the ratios 1:1, 1:2 and 1:3 (figure 54). The three components were incubated overnight at 4°C to enable the formation of the ternary complex. 50 ng of Lb^{pro} wt were used for cleavage at 30°C for 30 min. This experiment was mainly done to restore the larger cleavage product, which became very faint after

some assays (figure 41). The idea was that the addition of eIF4E and m⁷GTP might restore the hardly visible larger cleavage product (cp_N) running at about 18 kDa. Surprisingly, the smaller processing product running at about 11 kDa was detectable. Therefore, eIF4GII was processed by Lb^{pro} wt even though no uncleaved eIF4GII is present on the SDS gel.

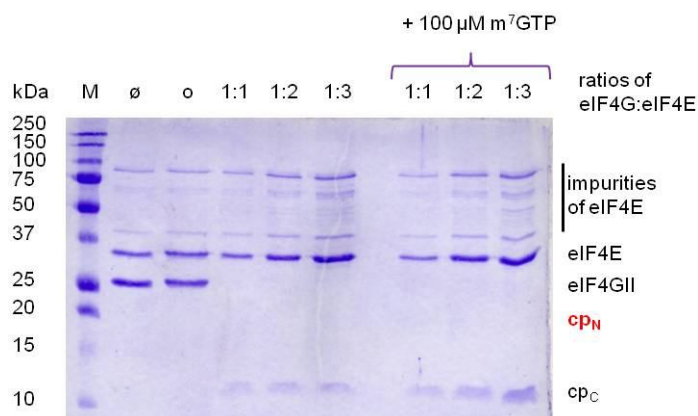


Figure 54: Processing assay with different ratios of eIF4GII:eIF4E and 50 ng Lb^{pro} wt. 50 ng of the protease were incubated for 10 min at 30°C with a 1:1, 1:2 or 1:3 ratio of eIF4GII:eIF4E without and with 100 μM m⁷GTP, respectively. ø and o represents the not incubated and incubated 1:1 ratio of eIF4GII:eIF4E. cp_N and cp_C indicate the larger and smaller processing products of eIF4GII, respectively. Impurities of the eIF4E sample run between 100-40 kDa. M indicates the Precision Plus Protein Standard All Blue from BioRad (7 μl loaded).

4.5 Observing the native state by blue native gel electrophoresis

In order to investigate the origin of the problems with the larger cleavage product (cp_N), obtained by cleavage of eIF4GII by the Lb^{pro} wt, the native state of the proteins was observed by means of blue native gel electrophoresis (BN-PAGE). Not only the processing assays but also the binary complex eIF4G:eIF4E and the ternary complex eIF4G:eIF4E with 100 μM m⁷GTP were analyzed (3.3.10). This should shed light on the one hand on the native state of eIF4GII. On the other hand, we wanted to observe the interaction of the different components of the complex. For the processing assay, 50 ng of Lb^{pro} wt were used to cleave eIF4GII at 30°C for 10-30 min. The cleavage reaction was stopped with 4 μl BN-sample buffer.

At first a 10% BN-PAGE was performed, but the percentage was too low to see the smaller cleavage fragment. Figure 55 shows the 10% BN-PAGE displaying the processing assay of eIF4GII with 50 ng of Lb^{pro} wt on the left and the binary (eIF4GII-eIF4E) and ternary (eIF4GII-eIF4E-m⁷GTP) complex formation on the right.

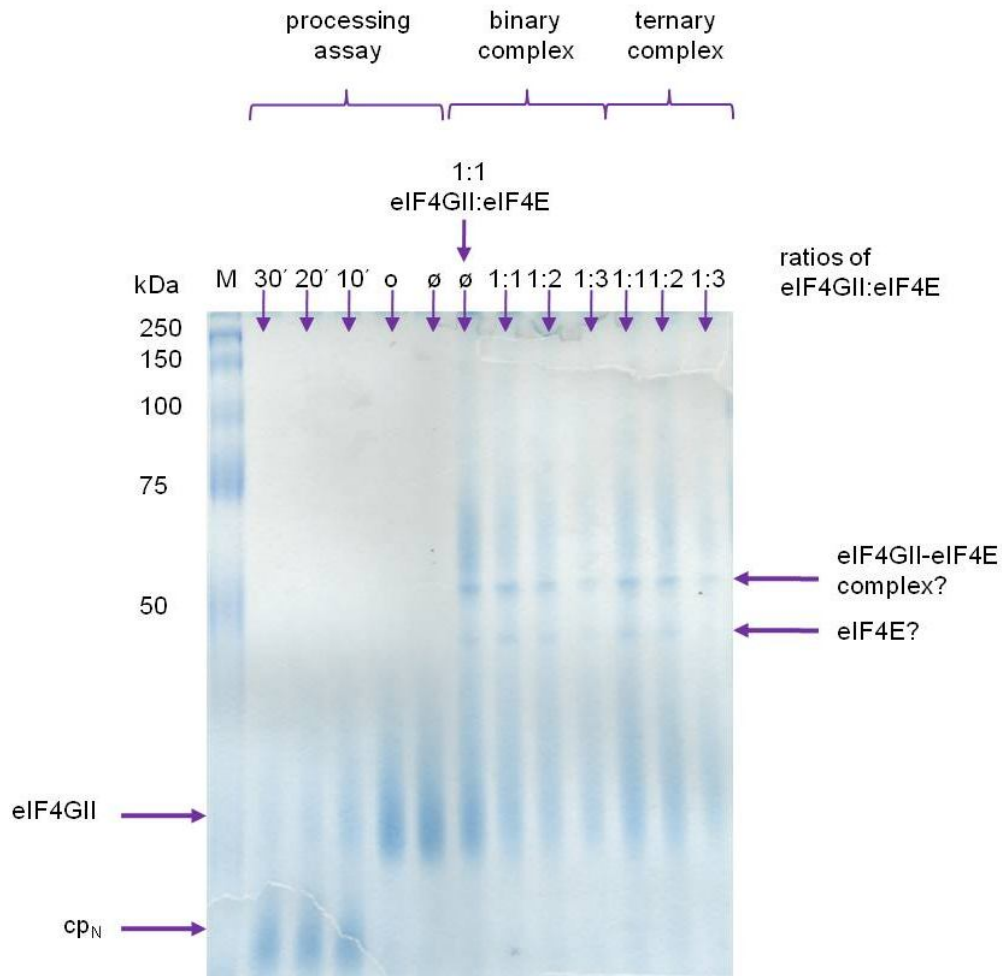


Figure 55: 10% BN-PAGE showing the processing assay and the complex formation. The processing assay of 50 ng Lb^{pro} wt with 3 µg eIF4GII is displayed at the left side of the gel. Incubation at 30°C and various times (min). At the right side, the binary complex of eIF4GII-eIF4E and the ternary complex eIF4GII-eIF4E-m⁷GTP are loaded. ∅ and o represent the not incubated and incubated negative control of eIF4GII for the processing assay. ∅ on the right side indicates the not incubated 1:1 complex of eIF4GII-eIF4E. cp_N indicates the larger processing product of eIF4GII. M indicates the Precision Plus Protein Standard All Blue from BioRad (12 µl loaded).

Therefore, a 15 % BN-PAGE was prepared (data not shown). The gel again did not run long enough to observe the smaller cleavage products. Nevertheless, the larger cleavage products were visible on both BN-PAGES. Therefore, we assumed that eIF4GII was still functional and hence in its native state. The formation of the binary or ternary complex was not confirmed explicitly by BN-PAGE. The eIF4GII fragment can be assigned to bands running at about 25 kDa. However, neither eIF4E nor the binary or ternary complex can be definitely identified on the BN-PAGE. To demonstrate the formation of the eIF4GII-eIF4E, complex various parameters for the BN-PAGE would need to be tested.

4.6 Complex formation of eIF4E with the eIF4GII fragment

The eIF4GII fragment we were working with has a theoretical molecular weight of about 22 kDa containing the amino acids 552-745 of wild-type human eIF4GII. This fragment contains the conserved eIF4E binding motif YXXXXLΦ as well as the cleavage sites recognized by the Lb^{pro} and HRV2 2A^{pro}. Mader *et al.* have documented the binding of different eIF4GII fragments to eIF4E wild-type *in vitro* (Mader *et al.* 1995). This property was investigated to confirm the hypothesis that binding of eIF4E to eIF4GII enhances the cleavage by Lb^{pro}.

Two different approaches were tested whether wild-type human eIF4E binds to the fragment of human eIF4GII. On the one hand, we tried to demonstrate the formation of the binary complex via size-exclusion chromatography using a HiLoad 16/60 Superdex 75 pg column (3.3.4). On the other hand, the property of eIF4E binding the 5' cap structure of mRNAs was exploited. Hence, 7-Methyl-GTP Sepharose 4B beads were used that are known to bind eIF4E. Thus, if eIF4GII binds to eIF4E both can be identified using 7-Methyl-GTP Sepharose 4B beads.

4.6.1 HiLoad 16/60 Superdex 75 prep grade gel filtration column

First, we tried to determine the presence of the complex using a HiLoad 16/60 Superdex 75 prep grade gel filtration column from GE Healthcare. To determine the approximate size of the proteins, the column was calibrated with a gel filtration low molecular weight calibration kit from GE Healthcare containing ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa) and ovalbumin (43 kDa). To determine the molecular weight by gel filtration, the elution volume parameter of the protein of interest is combined with elution volumes obtained for the standard proteins.

The proteins of interest have a theoretical molecular weight of 22 kDa and 25 kDa; thus, the complex should have a molecular weight of about 50 kDa. Before the start of the fraction collection, the loop was emptied with 5 ml and the column washed with 0.2 CVs ($\approx$ 24 ml of Buffer A). Figure 56 shows the chromatogram of the three standard proteins detected at 280 nm.

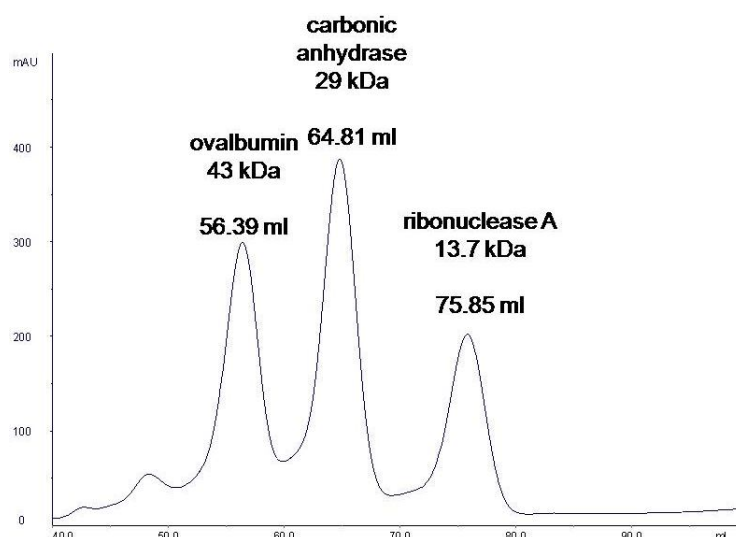


Figure 56: Chromatogram of the calibration of the HiLoad 16/60 75 µg size-exclusion column. The stock solutions of the calibration kit were diluted to 20 mg/ml as described in the manual. 3 mg of ribonuclease A, 4 mg of carbonic anhydrase and 4 mg of ovalbumin were used in a total volume of 1 ml (add Buffer A). Proteins were centrifuged at 9.700 rpm for 10 min at 4°C before loading via a 2 ml loop. The three standard proteins used for calibration, their molecular weight and their retention volume are indicated.

After the calibration, 1 mg of eIF4GII was loaded together with 2 mg of ribonuclease A, which was used as an internal standard (figure 57a).

As the yield of purified eIF4E was much lower, only 0.5 mg were loaded with 2 mg of ribonuclease A. The internal standard eluted again at the same elution volume. However, the peak of eIF4E (figure 57b) overlaps with some other component that could not be detected on the 17.5% SDS gel. Although, the recommendations are to load 1 mg of protein, 0.5 mg of eIF4E was sufficient to give an acceptable signal. To ensure the comparison between the different runs, the chromatograms obtained for eIF4GII and eIF4E (red curve) were overlaid with the chromatogram of the standard proteins (black curve) as shown in figure 57. For the overlay of two chromatograms, the ribonuclease peak of for example the chromatogram containing eIF4GII was oriented at the ribonuclease peak of the standard chromatogram.

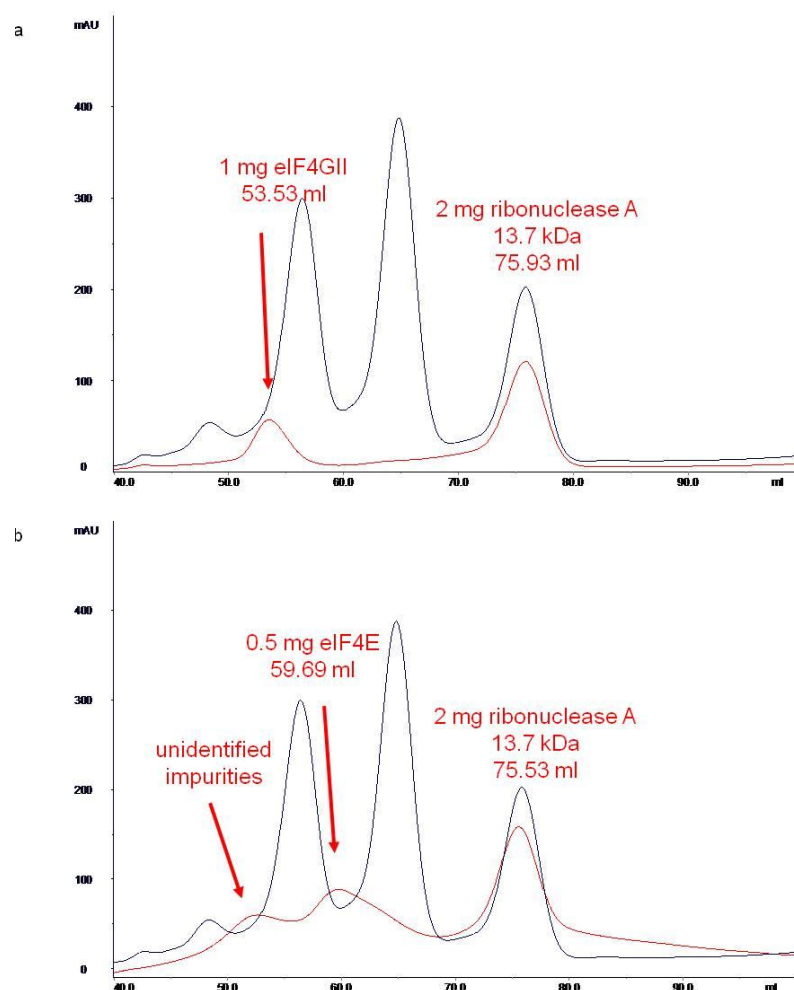


Figure 57: Overlay of the protein standards with either eIF4GII or eIF4E. (a) 1 mg of eIF4GII and 2 mg of ribonuclease A as internal standard were loaded on a 16/60 Hi Load Superdex 75 pg column. **(b)** 0.5 mg of eIF4E and 2 mg of ribonuclease A. The elution volumes are indicated. The black graph represents the standard curve, the red graph eIF4GII and eIF4E respectively.

According to Slepnev *et al.*, the optimal ratio of eIF4GII:eIF4E for complex formation is with equal or lower amounts of eIF4E (Slepnev *et al.* 2008). Hence, eIF4GII:eIF4E ratios of 1:1, 1:0.5 and 1:0.25 were incubated overnight at 4°C prior to size-exclusion chromatography. 0.5 mg of eIF4GII was used with 0.5 mg, 0.25 mg or 0.125 mg of eIF4E. 2 mg of ribonuclease A was added to each run as internal standard. Figure 58 presents the run of the 1:1 ratio of eIF4GII and eIF4E. The ribonuclease A peak as well as the peak of eIF4GII are visible at comparable elution volumes as seen in figure 57a. However, no signal for eIF4E could be detected, as none of the peaks would be at an equal elution volume as the one shown in figure 57b. Therefore, the formation of a complex between eIF4GII and eIF4E could not be demonstrated by size-exclusion. The chromatograms of the 1:0.5 and 1:0.25 ratios of eIF4GII:eIF4E are not shown as no signal for eIF4E was obtained.

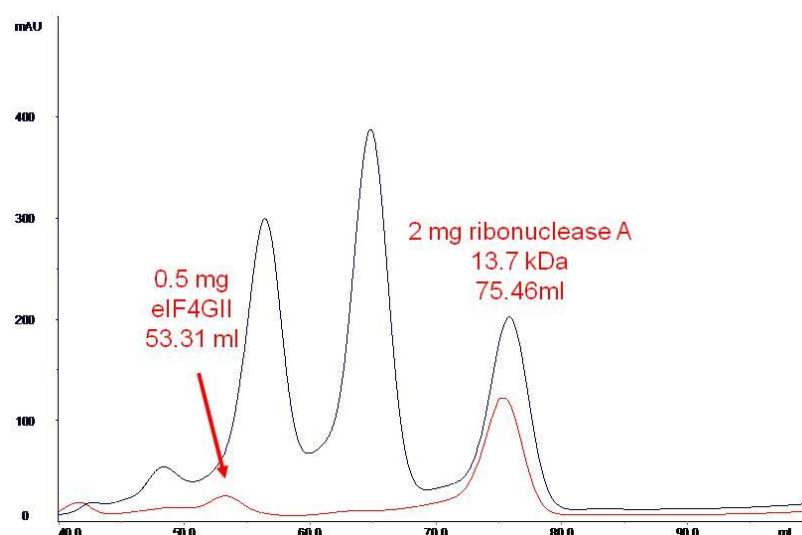


Figure 58: Overlay of the protein standards with the chromatogram of the the 1:1 ratio of eIF4GII:eIF4E. 0.5 mg of eIF4GII, 0.5 mg of eIF4E and 2 mg of ribonuclease A were loaded on a 16/60 HiLoad Superdex 75 pg column. The ribonuclease A peak and the peak of eIF4GII are indicated as well as their elution volume. The black graph indicates the standard curve, the red the curve of eIF4GII:eIF4E in a ratio of 1:1.

4.6.2 Binding to m⁷ GTP beads

The second approach to demonstrate complex formation of eIF4GII-eIF4E was by using 7-Methyl-GTP Sepharose 4B beads. As described in 3.3.4, the m⁷GTP beads were used to purify eIF4E. In the case of complex formation, eIF4E would bind to m⁷ GTP of the beads and eIF4GII to the bound eIF4E.

The beads were incubated overnight at 4°C with 500 µl of eIF4E (0.5 µg/µl). Unbound eIF4E was washed out with 6 ml of eIF4E binding buffer, as described in 3.3.4.3 2 ml fractions were collected and examined on a 17.5% SDS gel (data not shown). 200 µl eIF4GII (10 µg/µl) were added overnight at 4°C. A large excess of eIF4GII was chosen to ensure the binding to eIF4E on the beads. The unbound eIF4GII was washed out with 6 ml eIF4E binding buffer (20 mM MOPS pH 7.6, 0.25 mM DTT, 0.1 mM EDTA, 50 mM NaF and 100 mM KCl) and a 9 µl aliquot of the beads was analyzed on a 17.5% SDS gel (figure 59a). To elute eIF4E, the beads were incubated for 3 h with 600 µl of eIF4E elution buffer at 4°C. After the elution with 10 ml eIF4E elution buffer, the beads were regenerated as described in 3.3.4. The 1 ml fractions collected during the elution step were examined by SDS PAGE (figure 59b). Most of eIF4GII is washed out during the washing step. As the aliquot of the beads contains eIF4E and eIF4GII, we assume that wild-type eIF4E indeed binds to the here used eIF4GII fragment and builds a complex. Surprisingly, in the elution fractions (E1-E9) only eIF4GII and not eIF4E was observed. This might be due to the already low amount of eIF4E shown in the

beads aliquot and elution dilutes this even further thus it is no longer visible on the SDS PAGE.

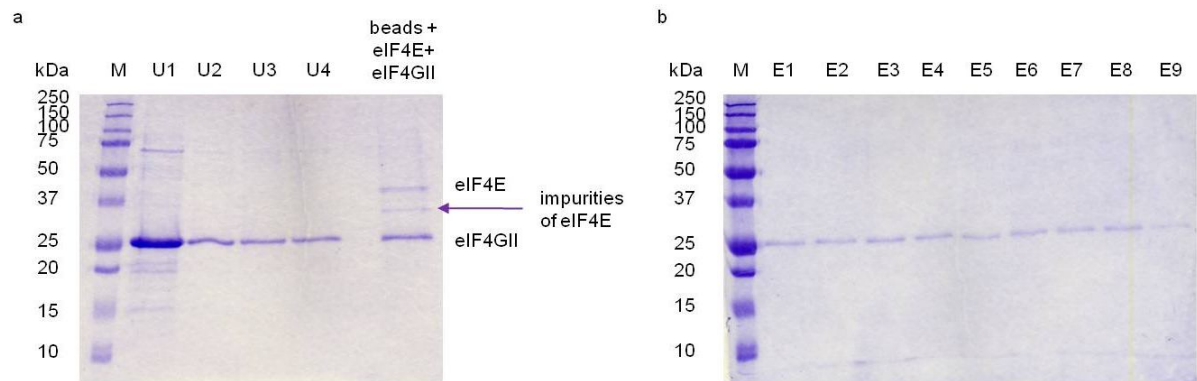


Figure 59: Complex formation confirmed using 7-Methyl-GTP Sepharose beads. (a) 9 μ l of each unbound eIF4GII fraction (2 ml fractions of U1-U4) and 9 μ l aliquot of beads with eIF4GII and eIF4E before incubation with eIF4E elution buffer. **(b)** After incubation with 500 μ l elution buffer, 1 ml elution fractions of eIF4E (E1-E9) were collected. M indicates the Precision Plus Protein Standard All Blue from BioRad (12 μ l loaded).

5 Discussion

As the leader protease of FMDV is an excellent target for antiviral drugs, the investigation of its binding properties is of great importance. The crucial step during the production of viable virus particles is the self-processing reaction of Lb^{pro}. During this reaction Lb^{pro} frees itself from the viral polyprotein by cleavage between its own C-terminus and the N-terminus of the capsid protein VP4. The inhibition of the self-processing event is thought to impair the formation of viable virions. Due to previous experiments on the 2A protease of poliovirus (Crowder *et al.* 2005), Lb^{pro} is considered to act similar and remain connected to VP4 which hinders correct capsid formation. Hence, an antiviral drug designed to prevent the self-processing of Lb^{pro} is thought to protect from FMDV infection.

Lb^{pro} is known to be a highly specific protease processing only three known substrates, the viral polyprotein and the two homologues of eIF4G (eIF4GI eIF4GII). A consensus sequence of these cleavage sites cannot be determined due to the strong variation amongst them. However, Lb^{pro} was found to prefer Lys at P₁ in the viral polyprotein whereas Gly is favored in eIF4GI and eIF4GII (Strebel *et al.* 1986, Foeger *et al.* 2002, Gradi *et al.* 2004). The main determinant of specificity is the S₂ subsite comprising a deep hydrophobic pocket accepting only Val, Leu and, to some extent, Ile. Surprisingly, a cleavage site in eIF4GII similar to the natural cleavage sequence in eIF4GII is not recognized by Lb^{pro}. The presence of Phe at P₂ and Asp at P₃ at this site inhibit cleavage due to sterical hindrance, mainly from Leu143 of the hydrophobic pocket (Mayer *et al.* 2008). The substitution of Leu143 with Ala provides more room in the S₂ pocket and allows Phe to be accepted. Therefore, Leu143 seems to occupy an important position in the hydrophobic pocket excluding substrates containing Phe at P₂. This property is further underlined by the fact that the only other residue found at position 143 amongst FMDV strains is Met.

To gain deeper insight in the binding specificities, a leader protease containing the L143A mutation was generated. The mutant protease was then compared to the wild-type regarding the cleavage properties on two different eIF4GII fragments. Lb^{pro} L143A was expected to recognize additional cleavage sites, thus producing additional cleavage products. For the identification of these cleavage sites mass spectrometry analysis was chosen. Furthermore, according to Ohlmann *et al.* 1997 the presence of eIF4E improves eIF4G processing by Lb^{pro}. eIF4G is thought to become structured upon binding eIF4E thus exposing the Lb^{pro} recognition site. These conformational changes may grant better access to the cleavage site for Lb^{pro}. Hence, the differences of cleavage in presence and absence of eIF4E were analyzed.

5.1 Complex formation of the eIF4GII fragment and eIF4E wild-type

Two different approaches were used to examine the binding of the used eIF4GII fragment to wild-type human eIF4E. The amino acid sequence of the eIF4GII fragment used throughout the thesis is shown in figure 24a.

A HiLoad 16/60 Superdex 75 prep grade gel filtration column was used to confirm the binding of eIF4E to the eIF4GII fragment. Due to physical problems with the column itself, some peaks show slight fronting. Nevertheless, ribonuclease A used as an internal standard elutes at comparable volumes in all runs as well as the eIF4GII sample and eIF4E during the single runs (figures 56 and 57). However, when different amounts of eIF4E:eIF4GII were preincubated overnight and loaded on the column, only peaks of eIF4GII and ribonuclease A were detectable, as confirmed by SDS PAGE. Even though the same amount of eIF4E was used as during the single run (figure 57b), no signal was obtained for eIF4E in complex with eIF4GII. The complex of eIF4E-eIF4GII should have a molecular weight of about 50 kDa and thus elute earlier than eIF4GII and eIF4E alone. No peak at an elution volume earlier than 52 ml was detected suggesting that no complex formation occurred or was below detection limit.

The determination of the molecular weight using size-exclusion chromatography is intended for globular proteins with a defined fold as Lb^{pro} wt. eIF4GII was shown to contain highly flexible regions and to undergo a unfolded-to-folded transition upon binding to the cap binding protein eIF4E (Hershey *et al.* 1999, Gross *et al.* 2003). This might be one reason that eIF4GII elutes not at the volume expected for a protein of 22 kDa. According to its theoretical size of 25 kDa, eIF4E would be expected to elute before eIF4GII. eIF4GII elutes at about 53 ml whereas eIF4E elutes at about 60 ml. This would argue again for partially unstructured region in eIF4GII influencing its elution.

The manual of the low molecular calibration kit suggests to load 1 mg of the protein of choice with 2 mg of an internal standard. As the yield of eIF4E was very low with the purification methods used, 1 mg of eIF4E could not be loaded. Consequently, another reason that eIF4E was not observed during any run together with eIF4GII might be the low amount of protein loaded. The fractions possibly containing protein were analyzed by SDS PAGE, but eIF4E was not detected in any run together with eIF4GII.

Thus, another method was chosen to confirm the interaction of eIF4GII and eIF4E. For the second attempt 7-Methyl-GTP Sepharose beads were used. The cap binding protein eIF4E binds to 7-Methyl-GTP. Additional eIF4GII thus should bind eIF4E and stay bound to the beads via eIF4E. First the beads were incubated with eIF4E overnight. After washing out unbound eIF4E, an excess of eIF4GII was added to the beads and

again incubated overnight. Following the washout of unbound eIF4GII, an aliquot of the beads was taken and analyzed together with the washout on a 17.5% SDS gel (figure 59a). Most of added eIF4GII was washed out during the washing step. However, some eIF4GII seems to stay bound to the beads via eIF4E. However, we cannot exclude that still some unbound eIF4GII is present. The separation of the beads aliquot show the bands of eIF4GII at 25 kDa and eIF4E at about 37 kDa. Another band at about 33 kDa arises from impurities of the eIF4E sample. Interestingly, the fractions collected during the elution of eIF4E show only eIF4GII. This might be due to the excess of eIF4GII added and the heavy dilution of eIF4E. Nevertheless, the eIF4GII fragment can be considered to bind wt eIF4E and thus forming a complex.

Instead of the eIF4E elution buffer used throughout this experiment, 0.75 μ M m^7 GTP can be added to a buffer containing 20 mM Hepes pH 7.6, 0.5 mM DTT, 0.2 mM EDTA, 10 % glycerol and 0.5 mM PMSF to elute eIF4E as performed by Zapata *et al.* 1993. 0.1 mM GTP can also be used instead of m^7 GTP. Furthermore, von der Haar *et al.* 2000 showed that eIF4E is also eluted using 20 mM GDP. Whether the use of m^7 GTP, GTP or GDP alters the amount of eIF4E after purification or yields a more definite result regarding the interaction between eIF4GII-eIF4E is not known. Hence, subsequent experiments would be needed to document a difference.

Similar experiments by Lamphear and colleagues using 7-Methyl-GTP Sepharose beads confirmed the interaction of the N-terminal fragment of eIF4GI to eIF4E (Lamphear *et al.* 1995). Subsequent studies by Mader *et al.* verified these findings (Mader *et al.* 1995). Furthermore, the eIF4GII fragment used throughout this thesis is thought to bind full-length eIF4E as comparable fragments of eIF4G were shown to bind full length mouse eIF4E (Mader *et al.* 1995). One prerequisite for efficient binding of eIF4G to eIF4E was shown to be the presence of the conserved eIF4E binding motif. However, fragments containing this sequence at the very N-terminus did not bind eIF4E. In addition, the presence of m^7 GTP, as present on the 7-Methyl-GTP beads, should further enhance the binding affinity of eIF4E to eIF4G.

Although these findings would argue for the interaction of the eIF4GII fragment with full-length human eIF4E, subsequent approaches would be needed to ensure the binding. Techniques like Far Western Blot as used in Mader *et al.* 1995 or other methods testing protein-protein interactions as co-immunoprecipitation or two-hybrid screening may be performed. However, the fact that unidentified regions important for eIF4E interaction might be lacking on the truncated eIF4GII needs to be considered as well.

5.2 Processing properties of Lb^{pro} wt and Lb^{pro} L143A on eIF4GII

Lb^{pro} wt and Lb^{pro} L143A were shown to be active by cleaving eIF4GII of HeLa cell extracts (figure 38). Consequently, both proteases were incubated with the eIF4GII fragment and the cleavage products analyzed. With 50 ng of both proteases, 50% of eIF4GII were cleaved after 10 min at 30°C. Furthermore, the kinetics of the cleavage reaction were very fast as the major differences are detectable in the first 10 min (figure 40 and 43).

As mentioned before, position 143 of the leader protease is important for the sustainment of substrate specificity by preventing the cleavage of Phe at P₂ sites. By mutation of this residue to Ala, more space is provided in the hydrophobic S₂ pocket of Lb^{pro}. Therefore, Lb^{pro} L143A is expected to cleave additional sites containing Gly at P₁ and Arg or Ser at P₁' as well as the related cleavage sequence in eIF4GII (AFADFGX^{red}RQTPGG) not recognized by Lb^{pro} wt. Indeed, additional cleavage products with Lb^{pro} L143A could be identified (figure 43) and were examined by mass spectrometry (figure 45). However, peptides matching for both samples did not give concrete information on additional cleavage sites. Interestingly, matches were only found for the very N- and C-terminal part of eIF4GII. The related sequence, expected to be cleaved by Lb^{pro} L143A, is also part of the peptides found by mass spectrometry. Still, it is not clearly distinguishable as additional cleavage site as only parts of the peptide which would be generated by cleavage at this sequence were detected by mass spectrometry. For exact determination of the cleavage sequences N-terminal sequencing might be the method of choice.

After several processing assays the larger cleavage product became weaker on SDS gels. To determine the reason, new aliquots of Lb^{pro} wt and eIF4GII as well as new buffer were used. Furthermore, eIF4E and m⁷GTP were added to the assays as this should improve the processing reaction. Neither of these trials could restore the larger cleavage product. Furthermore, to determine whether the proteins behave similarly in their native state, blue native gel electrophoresis was performed. Besides the processing assays, different ratios of the binary complex eIF4GII:eIF4E and the ternary complex eIF4GII:eIF4E:m⁷GTP were loaded to observe potential complex formation. The 10% BN PAGE (figure 55) shows that the larger cleavage product is present in amounts comparable to the cleavage products observed in figure 39. Hence, eIF4GII seems to be in the native state and successfully processed by Lb^{pro} wt. Due to its low percentage of the BN-PAGE, the smaller processing product is not detectable. The different ratios of eIF4GII:eIF4E with and without m⁷GTP did not shed light on the formation of the binary or ternary complex. As marker, the Precision Plus Protein Standard All Blue from BioRad

was loaded on the native gels even though it is recommended for SDS PAGE. Especially the marker bands of the 15% gel (data not shown) were not well distinguishable from each other, which makes the identification of the potential complex more difficult. Consequently, no conclusion concerning the complex of eIF4E-eIF4GII can be drawn using BN-PAGE.

To confirm the interaction between eIF4E and eIF4GII by BN-PAGE subsequent trials using different parameters would be necessary. However, the larger cleavage product, previously not detectable by SDS PAGE, was observed again in subsequent processing assays. The reason for the absence of the larger cleavage product could not be determined.

5.3 Processing properties of Lb^{pro} wt and Lb^{pro} L143A on eIF4GII^{self}

Besides the eIF4GII fragment, another fragment termed eIF4GII^{self} was used for several processing assays. In eIF4GII^{self}, the wild-type cleavage site of eIF4GII LLNVG↓SRRSQ was replaced by QRKLK↓GAGQS. The chemical environment present in eIF4GII is different to the one in Lb^{pro}. Therefore, we repeated the processing assays with eIF4GII^{self} as substrate and Lb^{pro} wt or Lb^{pro} L143A. The Lb^{pro} self-processing sequence was recognized by both proteases despite the differences in the chemical environment (figure 48 and 49). Surprisingly, the amount of both proteases needed to cleave 50% of eIF4GII^{self} was much lower than for eIF4GII. 15 ng of Lb^{pro} wt and 6.25 ng of Lb^{pro} L143A were sufficient to process 50% of eIF4GII^{self} at 30°C for 10 min. Despite the fact that the Lb^{pro} self-processing site is not present in wild-type eIF4GII, it is recognized with higher efficiency than the wild-type cleavage sequence. This leads to the assumption that Lb^{pro} has a greater affinity for the Lb^{pro} self-processing sequence than the cleavage sequence present in eIF4GII.

Santos *et al.* 2009 examined the hydrolytic activity of Lb^{pro} using peptides derived from the self-processing site and the cleavage sites on eIF4GI and eIF4GII. These peptides varied in length and sequence to examine the extended Lb^{pro} binding site. Subsequent experiments on the processing reactions with different peptides revealed that the specificity of Lb^{pro} is mainly determined by the nonprime side and dependent on the size of the substrate. Furthermore, Santos and colleagues demonstrated that peptides containing cleavage sites of eIF4GI or eIF4GII were poor substrates compared to peptides including the self-processing site. An explanation might be that the peptides used were missing two binding sites essential for processing by Lb^{pro}. One binding site allows the recruitment of eIF4E and the second was shown to be crucial for the binding

of the C-terminal extension of Lb^{pro}. The lack of these interactions could explain the poor cleavage in the *in vitro* assay. During my work, eIF4GII fragments containing both sites were used. However, my work confirms the results of Santos *et al.*, as the eIF4GII fragment comprising the Lb^{pro} self-processing site was cleaved more efficiently as the fragment with the natural cleavage site.

5.4 Change of processing properties of Lb^{pro} wt /Lb^{pro} L143A using additional eIF4E

In the course of cap-dependent translation eIF4G together with eIF4E and eIF4A forms the translation initiation complex eIF4F. While eIF4E binds the cellular cap of mRNA, eIF4G acts as scaffold binding eIF4E, the poly(A) tail and eIF3 which coordinates the complex to the 40S ribosomal subunit. Previous experiments demonstrated that eIF4G is cleaved more efficiently in the presence of eIF4E as an unfolded-to-folded transition of eIF4GII is proposed (Gross *et al.* 2003). Subsequently, the cleavage sequence should become better accessible to the protease enhancing the processing efficiency. The conformational changes eIF4G is able to undergo suggests that it might have different biochemical properties in the absence of eIF4E (Hershey *et al.* 1999). As most of these experiments displaying the unfolded-to-folded transition were only performed with truncated versions of eIF4G, further data on the structure of eIF4G in presence and absence of eIF4E is required.

To test whether the eIF4GII or eIF4GII^{self} fragment is processed more efficiently, eIF4E was added in different ratios to during the processing assays.

5.4.1 Processing of eIF4GII with eIF4E and Lb^{pro} wt/ Lb^{pro} L143A

Foremost, processing assays with eIF4GII:eIF4E in ratios of 1:1, 1:2 and 1:3 were performed using 50 ng of protease (figure 51). However, no significant differences between the processing assays with only eIF4GII and those in presence of eIF4E were observed. Furthermore, no enhanced cleavage effect can be seen between the different amounts of eIF4E added. Interestingly, the larger cleavage product (cp_N) in the assay of figure 51 runs differently depending on the protease used for cleavage. Lb^{pro} L143A seems to produce two larger cleavage products of similar size; one of the same size as the cp_N generated with Lb^{pro} wt, the other cleavage product slightly smaller. This might lead to the conclusion that Lb^{pro} L143A processes the related cleavage sequence AFADFG↓RQTPGG found in eIF4GII as well. However, this could not be verified by

mass spectrometry and similar assays using eIF4GII^{self} instead did not show this discrepancy.

According to Slepnev *et al.* 2008 cleavage of eIF4G is improved by adding equal or lower amounts of eIF4E to eIF4GII. Therefore, assays with ratios 1:1, 1:0.5 and 1:0.25 of eIF4GII:eIF4E and 50 ng of the protease were carried out (figure 52). Again, the size difference of the larger cleavage product generated with the mutant protease was present, but no improvement of cleavage could be detected. Other experiments testing the assembly of the 48S complex as in Morino *et al.* used as well lower amounts of eIF4E (0.3 µg) with 2 µg of eIF4G (Morino *et al.* 2000). This indicates again, that the binding of eIF4E to eIF4G prefers lower amounts of eIF4E.

Ohlmann *et al.* proposed that in the case of the leader protease, the eIF4G-eIF4E complex is a much better substrate than eIF4G alone (Ohlmann *et al.* 1997). In contrast to HRV2 2A^{pro} which requires the presence of eIF4E for efficient cleavage of eIF4GII, Lb^{pro} is able to cleave eIF4G on its own (Haghihat *et al.* 1996). Furthermore, a 98 amino acid region of yeast eIF4G was shown to undergo an unfolded-to-folded transition upon binding of eIF4E (Hershey *et al.* 1999). This region overlaps with the eIF4E binding sequence on eIF4G. Gross *et al.* confirmed these results and demonstrated that also the N-terminal region of yeast eIF4E (residues 24-35) becomes structured upon binding eIF4G (Gross *et al.* 2003). In turn, these residues promote the folding of amino acids 393-490 in yeast eIF4G. In human eIF4GII, the cleavage sequence for Lb^{pro} wt is located 66 amino acids distant from the conserved eIF4E binding motif. Hence, we speculated that the cleavage sequence recognized by Lb^{pro} also becomes structured upon binding of eIF4E. Due to the then structured region, the processing reaction might be enhanced as the region is better accessible for Lb^{pro}. However, no such effect was observed in any processing assay performed with eIF4GII and eIF4E. This might be because the eIF4GII fragment used is truncated and contains only the sites necessary for eIF4E binding and Lb^{pro} cleavage. As the conformational changes of eIF4G upon binding eIF4E have only been demonstrated in yeast so far, human eIF4GII might require additional regions or factors to become structured. Hence, further structural investigations on human eIF4G are needed to shed light on this process.

5.4.2 Processing of eIF4GII^{self} with eIF4E and Lb^{pro} wt /Lb^{pro} L143A

Processing assays using eIF4GII^{self} together with eIF4E were performed similar to those with eIF4GII. First eIF4E was used in higher ratios shown in figure 57. 6.25 ng of Lb^{pro} L143A and 15 ng of Lb^{pro} wt were used for the processing assay of eIF4GII^{self} as these amounts were shown to process 50% of eIF4GII^{self} after 10 min at 30°C. However,

with an excess of eIF4E no significant difference in cleavage was observed. Therefore, the amount of eIF4E was lowered and ratios of 1:1, 1:0.5 and 1:0.25 of eIF4GII^{self}:eIF4E were used. Again, no improvement of the cleavage ability of was identified. The different size of the larger cleavage product, as seen in processing assays of eIF4GII-eIF4E with both proteases, is not seen with eIF4GII^{self} as substrate. However, Lb^{pro} L143A clearly generates an additional cleavage product running at about 16 kDa which is not seen with Lb^{pro} wt.

5.4.3 Processing of eIF4GII with eIF4E and m⁷GTP

According to Niedzwiecka *et al.* 2002, eIF4G binding to eIF4E enhances the ability of eIF4E to interact with the mRNA 5' cap-structure and vice versa. Due to this suggestion 100 µM m⁷GTP were added to the processing assay containing eIF4GII:eIF4E in different ratios. This experiment was mainly performed to restore the larger cleavage product, which was observed to become weaker on SDS PAGE in several assays. No change in the processing pattern was recognizable. This might indicate that more m⁷GTP may be needed to observe an improved cleavage of eIF4GII.

5.5 Conclusion

The development of antiviral drugs is an important field of research not only regarding FMDV but also many other human and animal pathogens. In FMDV the leader protease is considered an optimal target for antiviral drugs. Previous experiments demonstrated that the inhibition of Lb^{pro} activity in cells causes a 1000-fold reduction in virus yield (Kleina *et al.* 1992). In addition, investigations on the 2A^{pro} of PV1 showed that if 2A^{pro} is inhibited, it remains connected to VP1 thus preventing the formation of viable virus (Crowder *et al.* 2005). These findings are thought to be also adoptable for Lb^{pro} of FMDV.

To design a functional inhibitor, information about the specificity and possible interactions of Lb^{pro} with substrates are necessary as is also the X-ray crystal structure. Several experiments indicate that Leu143 of Lb^{pro} is crucial for the maintenance of specificity. Leu143 is located in the S₂ pocket and prevents cleavage of substrates containing Phe at the P₂ position due to sterical hindrance. However, replacement of Leu143 by Ala provides more space and was shown to accept Phe (Mayer *et al.* 2008).

Therefore, a Lb^{pro} containing the L143A mutation was generated. As substrates, two different human eIF4GII fragments were used (aa 552-745). One, termed eIF4GII, contained the natural cleavage site recognized by Lb^{pro}. In the second fragment,

eIF4GII^{self}, the natural recognition sequence was replaced by the Lb^{pro} self-processing site. The different specificities were observed using Lb^{pro} wt and Lb^{pro} L143A with both substrates in processing assays. In the case of eIF4GII, 50 ng of both proteases were needed to process 50% of eIF4GII. In contrast, 15 ng of Lb^{pro} wt and 6.25 ng of Lb^{pro} L143A were sufficient for 50% cleavage of eIF4GII^{self}. The lower amount of protease needed to cleave eIF4GII^{self} suggests that the Lb^{pro} self-processing site might be recognized more efficiently despite the different chemical environment present in eIF4GII. Additionally, Lb^{pro} L143A generated aberrant processing products due to its lower specificity. However, analysis by mass spectrometry of two additional cleavage products did not reveal further cleavage sites recognized by Lb^{pro} L143A. To determine putative cleavage sites recognized by Lb^{pro} L143A, another method might be more effective.

In several processing assays eIF4E was added to eIF4GII. According to Gross *et al.* 2003, eIF4G undergoes an unfolded-to-folded transition upon binding to eIF4E. We hypothesize that also the recognition site for Lb^{pro} becomes more structured and exposed which might facilitate the cleavage by Lb^{pro}. Therefore, processing assays with supplementary eIF4E are thought to enhance the cleavage properties of Lb^{pro}. However, no significant difference in processing, neither with Lb^{pro} wt nor with Lb^{pro} L143A, was observed by adding eIF4E to the assays. This could be due to the use of human eIF4G and eIF4E, as most studies on enhanced processing were done in yeast (Hershey *et al.* 1999). A second explanation might be that the eIF4GII fragments are lacking certain unidentified regions important for eIF4E interaction and processing by Lb^{pro}.

6 Appendix

6.1 Abbreviations

4E-BP	4E-binding protein
aa	Amino acids
Amp	Ampicillin
BN PAGE	Blue native gel electrophoresis
Cam	Chloramphenicol
CIP	Calf intestine phosphatase
<i>cre</i>	Cis-acting RNA replication element
CTE	C-terminal extension
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eIF3	Eukaryotic initiation factor 3
eIF4A	Eukaryotic initiation factor 4A
eIF4E	Eukaryotic initiation factor 4E
eIF4F	Eukaryotic initiation factor 4F
eIF4G	Eukaryotic initiation factor 4GI / 4GII
FMDV	Foot-and-mouth disease virus
GDP	Guanosine dipohsphate
GTP	Guanosine triphosphate
HEAT	H untingtin, e longation factor 3, protein phosphatase 2A and yeast PI3 kinase TOR1
HAV	Hepatitis A virus
HeLa	Henrietta Lacks' cells
HRV	Human rhinovirus
ICAM-1	Intercellular adhesion molecule 1
ICTV	International committee on taxonomy of viruses
IFN	Interferon
IRES	Internal ribosomal entry segment
kDa	Kilo Dalton
LB	Luria Bertani
Lab ^{pro}	Leader protease ab
Lb ^{pro}	Leader protease b
mRNA	Messenger RNA

NF-κB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PABP	Poly-A binding protein
PV	Poliovirus
RGD	Arg-Gly-Asp
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SDS PAGE	Sodiumdodecylsulfate Polyacrylamide Gel electrophoresis
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
UTR	Untranslated region
UV	Ultra-violet
VP1-VP4	Viral protein 1-4
VPg	Viral protein genome linked
wt	Wild-type

6.2 List of amino acids

G	Gly	Glycine
A	Ala	Alanine
V	Val	Valine
L	Leu	Leucine
I	Ile	Isoleucine
M	Met	Methionine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
C	Cys	Cysteine
H	His	Histidine
F	Phe	Phenylalanine
Y	Tyr	Tyrosine
W	Trp	Tryptophane
N	Asn	Asparagine
Q	Gln	Glutamine
D	Asp	Aspartate
E	Glu	Glutamate
K	Lys	Lysine
R	Arg	Arginine

6.3 References

- Acharya, R., E. Fry, D. Stuart, G. Fox, D. Rowlands, and F. Brown.** 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. *Nature* **337**:709–716.
- Agol, V. I., A. V. Paul, and E. Wimmer.** 1999. Paradoxes of the replication of picornaviral genomes. *Virus Res.* **62**:129–147.
- Andino, R., G. E. Rieckhof, and D. Baltimore.** 1990. A functional ribonucleoprotein complex forms around the 5' end of poliovirus RNA. *Cell* **63**:369–380.
- Andino, R., G. E. Rieckhof, P. L. Achacoso, and D. Baltimore.** 1993. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5' end of viral RNA. *EMBO J.* **12**:3587–3598.
- Balvay, L., M., Lopez Lastra, B., Sargueil, J. L., Darlix, and T., Ohlmann.** 2007. Translational control of retroviruses. *Nat Rev Microbiol.* **5**(2): 128–140.
- Barton, D.J., Morasco, B.J. and Glanegan, J.B.** 1999. Translating ribosomes inhibit poliovirus negative-strand RNA synthesis *J. Virol.* **73**: 10104-10112.
- Barton, D. J., B. J. O'Donnell, and J. B. Flanagan.** 2001. 5' cloverleaf in poliovirus RNA is a cis-acting replication element required for negativestrand synthesis. *EMBO J.* **20**:1439–1448.
- Basavappa, R., R. Syed, O. Flore, J. P. Icenogle, D. J. Filman, and J. M. Hogle.** 1994. Role and mechanism of the maturation cleavage of VP0 in poliovirus assembly: structure of the empty capsid assembly intermediate at 2.9 Å resolution. *Protein Sci.* **3**:1651–1669.
- Baxt, B.** 1987. Effect of lysosomotropic compounds on early events in foot-and-mouth disease virus replication. *Virus Res.* **7**:257–271.
- Baxt, B., and Y. Becker.** 1990. The effect of peptides containing the arginine-glycine-aspartic acid sequence on the adsorption of foot-and-mouth disease virus to tissue culture cells. *Virus Genes* **4**:73–83.
- Bedard, K. M., and B. L. Semler.** 2004. Regulation of picornavirus gene expression. *Microbes Infect.* **6**:702-13.
- Belsham, G. J., and J. K. Brangwyn.** 1990. A region of the 5' noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. *J. Virol.* **64**:5389–5395.
- Belsham, G. J., and E. Martínez-Salas.** 2004. Genome organisation, translation and replication of foot-and-mouth disease virus RNA. In *Foot and Mouth Disease: Current Perspectives*, pp. 19–52. Edited by E. Domingo & F. Sobrino. Norfolk, UK: Horizon Bioscience.
- Berger, A., and I. Schechter.** 1970. Mapping the active site of papain with the aid of peptide substrates and inhibitors. *Philos. Trans. R. Soc. London B* **257**:249–264.
- Berinstein, A., M. Roivainen, T. Hovi, P. W. Mason, and B. Baxt.** 1995. Antibodies to the vitronectin receptor (integrin alpha V beta 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. *J. Virol.* **69**:2664–2666.
- Bienz, K., D. Egger, M. Troxler, and L. Pasamontes.** 1990. Structural organization of poliovirus RNA replication is mediated by viral proteins of the P2 genomic region. *J. Virol.* **64**:1156–1163.
- Birtley, J. R., S. R., Knox, A. M., Jaulent, P., Brick, R. J. Letherbarrow, and S. Curry.** 2005. Crystal structure of foot-and-mouth disease virus 3C protease. New insights into catalytic mechanism and cleavage specificity. *J Biol Chem.* **25**:11520-7.

- Brown, C. J., I., McNae, P. M., Fischer, and M. D. Walkinshaw.** 2007. Crystallographic and mass spectrometric characterisation of eIF4E with N7-alkylated cap derivatives. *J Mol Biol*, **372**: 7-15.
- Burke, K. L., J. W., Almond, and D. J. Evans.** 1991. Antigen chimeras of poliovirus. *Prog Med Virol* **38**: 56-68.
- Burman, A., S., Clark, N. G., Abrescia, E. E., Fry, D. I., Stuart, and T. Jackson.** 2006. Specificity of the VP1 GH loop of foot-and-mouth disease virus for alphav integrins. *J. Virol.* **80**:9798–9810.
- Cao, X., I. E. Bergmann, R. Fullkrug, and E. Beck.** 1995. Functional analysis of the two alternative translation initiation sites of foot-and-mouth disease virus. *J. Virol.* **69**:560–563.
- Carrillo, E. C., C. Giachetti, and R. Campos.** 1985. Early steps in FMDV replication: further analysis on the effects of chloroquine. *Virology* **147**: 118–125.
- Carrillo, C., E. R., Tulman, G., Delhon, Z., Lu, A., Carreno, A., Vagnozzi, G. F., Kutish, and D. L. Rock.** 2005. Comparative genomics of foot-and-mouth disease virus. *J. Virol.* **79**: 6487–6504.
- Cencic, R.,C., Mayer, M. A., Juliano, L., Juliano, R., Konrat, G., Kontaxis, and T., Skern.** 2007. Investigating the substrate specificity and oligomerisation of the leader protease of foot-and-mouth disease virus using NMR. *J. Mol. Biol.*, **373**:1071-87.
- Chinsangaram, J., M., Koster, and M. J. Grubman.** 2001. Inhibition of L-deleted foot-and-mouth disease virus replication by alpha/beta interferon involves double-stranded RNA-dependent protein kinase. *J. Virol.* **12**: 5498–5503.
- Crowder, S., and K. Kirkegaard.** 2005. Trans-dominant inhibition of RNA viral replication can slow growth of drug-resistant viruses. *Nat Genet.* **37**: 701-9.
- Cui, T., S. Sankar, and A. G. Porter.** 1993. Binding of encephalomyocarditis virus RNA polymerase to the 3'-noncoding region of the viral RNA is specific and requires the 3'-poly(A) tail. *J. Biol. Chem.* **268**:26093–26098.
- Cui, T., and A. G. Porter.** 1995. Localization of binding site for encephalomyocarditis virus RNA polymerase in the 3'-noncoding region of the viral RNA. *Nucleic Acids Res.* **23**:377–382.
- Curry, S., E. Fry, W. Blakemore, R. Abu-Ghazaleh, T. Jackson, A. King, S. Lea, J. Newman, and D. Stuart.** 1997. Dissecting the roles of VP0 cleavage and RNA packaging in picornavirus capsid stabilization: the structure of empty capsids of foot-and-mouth disease virus. *J. Virol.* **71**:9743–9752.
- de Felipe, P., L. E., Hughes, M. D., Ryan, and J. D. Brown.** 2003. Co-translational, intraribosomal cleavage of polypeptides by the foot-and-mouth disease virus 2A peptide. *J. Biol. Chem.* **278**:11441-11448
- DeLano D. L.** 2002. The PyMOL molecular graphics system.
- de los Santos, T., S., de Avila Botton, R., Weiblen, and M. J. Grubman.** 2006. The leader proteinase of foot-and-mouth disease virus inhibits the induction of beta interferon mRNA and blocks the host innate immune response. *J. Virol.* **80**: 1906–1914.
- de los Santos, T., Diaz-San Segundo, F. D., and M. J. Grubman.** 2007. Degradation of nuclear factor kappa B during foot-and-mouth disease virus infection. *J. Virol.* **81**:12803-12815.
- Diez, J., M., Ishikawa, M., Kaido, and P. Ahlquist.** 2000. Identification and characterization of a host protein required for efficient template selection in viral RNA replication. *Proc. Natl. Acad. Sci. USA* **97**: 3913–3918.

Donnelly, M. L., G. Luke, A. Mehrotra, X. Li, L. E. Hughes, D. Gani, and M. D. Ryan. 2001. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip.' *J. Gen. Virol.* **82**:1013–1025.

Escarmis, C., J. Dopazo, M. Davila, E. L. Palma, and E. Domingo. 1995. Large deletions in the 5'-untranslated region of foot-and-mouth disease virus of serotype C. *Virus Res.* **35**:155–167.

Etchison, D., S. C. Milburn, I. Ederly, N. Sonenberg, and J. W. Hershey. 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eukaryotic initiation factor 3 and a cap-binding protein complex. *J. Biol. Chem.* **257**:14806–10.

expasy.org. www.expasy.org

Fenner, F. J., P. J. Gibbs, F. A. Murphy, R. Rott, M. J. Studdert, and D. O. White. 1993. *Veterinary virology*, p. 403–430. Academic Press, New York, N.Y.

Ferrer-Orta, C., R., Agudo, E., Domingo, and N. Verdaguer. 2009. Structural insights into replication initiation and elongation processes by the FMDV RNA-dependent RNA polymerase. *Curr Opin Struct Biol.* **19**:752–8.

Fields, B. N., D. M., Knipe, and P. M., Howley. 2007. *Field's virology*. Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia, Pa.; London.

Foeger, N., W., Glaser, and T., Skern. 2002. Recognition of eukaryotic initiation factor 4G isoforms by picornaviral proteinases. *J Biol Chem.* **277**:44300–44309.

Foeger, N., E., Kuehnel, R., Cencic, and T., Skern. 2005. The binding of foot-and-mouth disease virus leader proteinase eIF4GI involves conserved ionic interactions. *FEBS J.* **272**:2602–2611.

Fox, G., N. R. Parry, P. V. Barnett, B. McGinn, D. J. Rowlands, and F. Brown. 1989. The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). *J. Gen. Virol.* **70**:625–637.

Fracastorius, H. 1546. *De sympathia et antipathia rerum liber unus. De contagione et contagiosis morbis et eorum curatione liber I*, Venice, Heirs of L. A. Junta.

Gamarnik, A. V., and R. Andino. 1998. Switch from translation to RNA replication in a positive-stranded RNA virus. *Genes Dev.* **12**:2293–2304.

George, M., R. Venkataramanan, C. B., Gurumurthy, and D., Hemadri. 2001. The non-structural leader protein gene of foot-and-mouth disease virus is highly variable between serotypes. *Virus Genes* **22**:271–278.

Gerber, K., E. Wimmer, and A. V. Paul. 2001. Biochemical and genetic studies of the initiation of human rhinovirus 2 RNA replication: purification and enzymatic analysis of the RNA-dependent RNA polymerase 3D(pol). *J Virol* **75**: 10969–78.

Glaser, W., and T., Skern. 2000. Extremely efficient cleavage of eIF4G by picornaviral proteinases L and 2A *in vitro*. *FEBS Lett* **480**: 151–155

Glaser, W., R., Cencic, and T. Skern. 2001. Foot-and-mouth disease virus leader proteinase: involvement of C-terminal residues in self-processing and cleavage of eIF4GI. *J. Biol. Chem.*, **276**:35473–35481.

Goodfellow, I., Y. Chaudhry, A. Richardson, J. Meredith, J. W. Almond, W. Barclay, and D. J. Evans. 2000. Identification of a *cis*-acting replication element within the poliovirus coding region. *J. Virol.* **74**:4590–4600.

Gradi, A., N., Foeger, R., Strong, Y. V. Svitkin, N., Sonenberg, T., Skern, and G. J. Belsham. 2004. Cleavage of eukaryotic translation initiation factor 4GII withing foot-and-mouth disease virus-infected cells: identification of the L-protease cleavage site in vitro. *J. Virol.* **78**: 3271-8.

Gradi, A., Imataka, H., Svitkin, Y. V., Rom, E., B. Raught, B., Morino, S. and N. Sonenberg. 1998. A novel functional human eukaryotic translation initiation factor 4G. *Mol Cell Biol*, **18**:334-342.

Gross, J. D., N. J., Moerke, T., von der Haar, A. A., Lugovskoy, A. B., Sachs, J. E., McCarthy, and G., Wagner. 2003. Ribosome loading onto the mRNA cap is driven by conformational coupling between eIF4G and eIF4E. *Cell* **115**:739–750.

Grubman, M. J., and H. L. Bachrach. 1979. Isolation of foot-and-mouth disease virus messenger RNA from membrane-bound polyribosomes and characterization of its 5' and 3' termini. *Virology* **98**:466–470.

Guarné, A., J. Tormo, R. Kirchweger, D. Pfistermueller, I. Fita, and T. Skern. 1998. Structure of the foot-and-mouth disease virus leader protease: a papain-like fold adapted for self-processing and eIF4G recognition. *EMBO J.* **17**:7469–7479.

Guarné, A., B., Hampoelz, B., Glaser, W., Caerpena, X., Tormo, J., Fita, I., and T. Skern. 2000. Structural and biochemical features distinguish the foot-and-mouth disease virus leader proteinase from other papain-like enzymes. *J. Mol. Biol.*, **302**:1227-1240.

Haghighat, A., and N., Sonenberg. 1997. eIF4G dramatically enhances the binding of eIF4E to the mRNA 5'-cap structure. *J Biol Chem*, **272**:21677-21680.

Harris, K. S., W. Xiang, L. Alexander, W. S. Lane, A. V. Paul, and E. Wimmer. 1994. Interaction of poliovirus polypeptide 3CDpro with the 5' and 3' termini of the poliovirus genome. Identification of viral and cellular cofactors needed for efficient binding. *J. Biol. Chem.* **269**:27004–27014.

Hentze, M. W. 1997. eIF4G: a multipurpose ribosome adapter? *Science*, **257**:500-501.

Herold, J., and R. Andino. 2000. Poliovirus requires a precise 5' end for efficient positive-strand RNA synthesis. *J. Virol.* **74**:6394–6400.

Herold, J., and R. Andino. 2001. Poliovirus RNA replication requires genome circularization through a protein-protein bridge. *Mol. Cell* **7**:581–591.

Hershey, P. E., S. M., McWhirter, J. D., Gross, G., Wagner, T., Alber, and, A. B., Sachs. 1999. The Cap-binding protein eIF4E promotes folding of a functional domain of yeast translation initiation factor eIF4G1. *J Biol Chem* **274**:21297–21304.

Hershey, J. W., and W. C. Merrik. 2000. The pathway and mechanism of initiation of protein synthesis. In Sonenberg, N., Hershey, J. W. and Mathews, M. (eds.), *Translational control of gene expression*. Cold Spring Harbor Laboratory Press, New York, pp. 33-88.

Hindiyeh, M., Q. H. Li, R. Basavappa, J. M. Hogle, and M. Chow. 1999. Poliovirus mutants at histidine 195 of VP2 do not cleave VP0 into VP2 and VP4. *J. Virol.* **73**:9072–9079.

Hogle, J. M., M., Chow, and D. J. Filman. 1985. Three-dimensional structure of poliovirus at 2.9 Å resolution. *Science*, **229**: 1358-65.

ICTV 2009 www.ictvonline.org International committee on taxonomy of viruses.

Imatka, H., A. Gradi, and N. Sonenberg. 1998. A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *Embo J.* **17**:7480-9.

Isaksson, A., M., Berggren, and A., Ricksten. 2003. Epstein-Barr virus U leader exon contains an internal ribosome entry site. *Oncogene*, **22(4)**: 572–581.

Jackson, T., F. M. Ellard, R. A. Ghazaleh, S. M. Brookes, W. E. Blakemore, A. H. Corteyn, D. I. Stuart, J. W. Newman, and A. M. King. 1996. Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. *J. Virol.* **70**:5282–5287.

Jackson, T., D. Sheppard, M. Denyer, W. Blakemore, and A. M. King. 2000. The epithelial integrin $\alpha\beta 6$ is a receptor for foot-and-mouth disease virus. *J. Virol.* **74**:4949–4956.

Jackson, T., A. P. Mould, D. Sheppard, and A. M. King. 2002. Integrin $\alpha\beta 1$ is a receptor for foot-and-mouth disease virus. *J. Virol.* **76**:935–941.

Jackson, T., A. M. King, D. I. Stuart, and E. Fry. 2003. Structure and receptor binding. *Virus Res.* **91**:33–46.

Jacobson, M. F., and D. Baltimore. 1968. Polypeptide cleavages in the formation of poliovirus proteins. *Proc. Natl. Acad. Sci. USA* **61**:77–84.

Jan, E. Divergent IRES elements in invertebrates. 2006. *Virus Res*, **119(1)**:16–28.

Ji, H., C. S., Fraser, Y., Yu, J., Leary, and J. A., Doudna. 2004. Coordinates assembly of human translation initiation complexes by hepatitis C virus internal ribosome entry site RNA. *Proc Natl Acad Sci USA*, **101**:16990-16995.

Jünemann, C., Y., Song, G., Bassili, D., Goergen, J., Henke, and M. Niepmann. 2006. Picornavirus Internal Ribosome Entry Site Elements Can Stimulate Translation of Upstream Genes. *Genes Dev.* **15**:1593-612.

Kamphuis, I. G., K. H., Kalk, M. B., Swarte, and J. Drenth. 1984. Structure of papain refined at 1.65 Å resolution. *J Mol Biol.* **179**: 233-56.

Kirchweger, R., E., Ziegler, B. J., Lamphear, D., Waters, H. D., Liebig, W., Sommergruber, F., Sobrino, C., Hohenadl, D., Blaas, R. E., Rhoads, and T., Skern. 1994. Foot-and-mouth disease virus leader proteinase: Purification of the Lb form and determination of its cleavage site on eIF-4gamma. *J. Virol.*, **68**:5677–5684.

Kleina, L. G., and M. J. Grubman. 1992. Antiviral effects of a thiol protease inhibitor on foot-and-mouth disease virus. *J. Virol.* **66**:7168–7175.

Kuehnel, E., R., Cencic, N., Foeger, and T., Skern. 2004. Foot-and-mouth disease virus leader proteinase: Specificity at the P2 and P3 positions and comparison with other papain-like enzymes. *Biochemistry.* **43**:11482-11490.

Kuhn, R., N. Luz, and E. Beck. 1990. Functional analysis of the internal translation initiation site of foot-and-mouth disease virus. *J. Virol.* **64**:4625– 4631.

Lamphear, B. J., R. Kirchweger, T. Skern, and R. E. Rhoads. 1995. Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. *J. Biol. Chem.* **270**:21975– 21983.

LeFebvre, A. K., Korneeva, N. L. Trutschl, M., Cvek, U., Duzan, R. D., Bradley, C. A., Bradley, C. A., Hershey, J. W., and R. E. Rhoads. 2006. Translation initiation factor eIF4G-1 binds to eIF3 through the eIF3e subunit. *J Biol Chem*, **281**:22917-22932.

Leippert, M., E. Beck, F. Weiland, and E. Pfaff. 1997. Point mutations within the βG - βH loop of foot-and-mouth disease virus O1K affect virus attachment to target cells. *J. Virol.* **71**:1046–1051.

Lescar, J., and B. Canard. 2009. RNA-dependent RNA polymerases from flaviviruses and Picornaviridae. *Curr Opin Struct Biol*, **19**: 759-67.

Lloyd, R. E., H. Toyoda, D., Etchison, E., Wimmer, and E., Ehrenfeld. 1986. Cleavage of the cap binding protein complex polypeptide p220 is not effected by the second poliovirus protease 2A. *Virology* **150**:299–303.

Lloyd, R. E. 2006. Translational control by viral proteinases. *Virus Res.* **119**:76-88.

Loeffler, F., and P. Frosch. 1897. Summarischer Bericht uber die Ergebnisse der Untersuchungen zur Erforschung der Maul- und Klauenseuche. *Zentbl. Bakteriol. Parasitenkd Abt. I* **22**:257–259.

Lopez de Quinto, S., M. Saiz, D. de la Morena, F. Sobrino, and E. Martinez-Salas. 2002. IRES-driven translation is stimulated separately by the FMDV 3'-NCR and poly(A) sequences. *Nucleic Acids Res.* **30**:4398–4405.

Lopez-Lastra, M., Rivas, A., and M.I. Barria. 2005. Protein synthesis in eukaryotes: the growing biological relevance of cap-independent translation initiation. *Biol Res* **38**:121-146.

Macejak, D.G., and P. Sarnow. 1991. Internal initiation of translation mediated by the 5' leader of a cellular mRNA. *Nature.* **353**: 90-4.

Mader, S., H. Lee, A. Pause, and N. Sonenberg. 1995 The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressor 4E-binding proteins. *Mol. Cell. Biol.* **15**:4990-7.

Marcotrigiano, J., A., Gingras, N., Sonenberg, and S. K., Burley. 1997. Cocystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. *Cell*, **89**:951-61.

Marcotrigiano, J., Lomakin, I.B., Sonenberg, N., Pestova, T., Hellen, C.U.T., and Burley, S.K. (2001). A conserved HEAT domain within eIF4G directs assembly of the translation initiation machinery. *Mol. Cell* **7**:193–203.

Marcotrigiano, J., A., Gingras, N., Sonenberg, and S. K., Burley. 1999. Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. *Mol.Cell*, **3**:707-716.

Marcotrigiano, J., Lomakin, I.B., Sonenberg, N., Pestova, T., Hellen, C.U.T., and Burley, S.K. 2001. A conserved HEAT domain within eIF4G directs assembly of the translation initiation machinery. *Mol. Cell* **7**:193–203.

Martínez-Salas, E., R., Ramos, E. Lafuente, and S. López de Quinto. 1999. Internal ribosome entry site biology and its use in expression vectors. *Curr Opin Biotechnol.***10**:458–64.

Mason, P. W., E. Rieder, and B. Baxt. 1994. RGD sequence of foot-and mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody-dependent enhancement pathway. *Proc. Natl. Acad. Sci. USA* **91**:1932–1936.

Mason, P. W., S. V. Bezborodova, and T. M. Henry. 2002. Identification and characterization of a *cis*-acting replication element (*cre*) adjacent to the internal ribosome entry site of foot-and-mouth disease virus. *J. Virol.* **76**: 9686–9694.

Mason, P.W., M. J. Grubman, and B. Baxt. 2003. Molecular basis of pathogenesis of FMDV. *Virus Res.* **91**: 9-32.

Matsuo H, H., Li, A. M., McGuire, C. M., Fletcher, A. C., Gingras, N., Sonenberg, and G., Wagner. 1997. Structure of translation factor eIF4E bound to m7GDP and interaction with 4E-binding protein. *Nat. Struct. Biol.* **4**:717–724

Mayer, C., D., Neubauer, A. T., Nchinda, R., Cencic, K., Tropf, and T., Skern. 2008. Residue L143 of the foot-and-mouth disease virus leader proteinase is a determinant of cleavage specificity. *J. Virol.*, **82**:4656-4659.

McKenna, T. S., J. Lubroth, E. Rieder, B. Baxt, and P. W. Mason. 1995. Receptor binding site-deleted foot-and-mouth disease (FMD) virus protects cattle from FMD. *J. Virol.* **69**:5787–5790.

McKnight, K. L., and S. M. Lemon. 1998. The rhinovirus type 14 genome contains an internally located RNA structure that is required for viral replication. *RNA* **4**:1569–1584.

Melchers, W. J., J. G. Hoenderop, H. J. Bruins Slot, C. W. Pleij, E. V. Pilipenko, V. I. Agol, and J. M. Galama. 1997. Kissing of the two predominant hairpin loops in the coxsackie B virus 3' untranslated region is the essential structural feature of the origin of replication required for negative-strand RNA synthesis. *J. Virol.* **71**:686–696.

Monaghan, P., H., Cook, T., Jackson, M. Ryan, and T. Wileman. 2004. The ultrastructure of the developing replication site in foot-and-mouth disease virus infected BHK-38 cells. *J Gen Virol.* **85**:993-46.

Moore, D. M., and K. M. Cowan. 1978. Effect of trypsin and chymotrypsin on the polypeptides of large and small plaque variants of foot-and-mouth disease virus: relationship to specific antigenicity and infectivity. *J. Gen. Virol.* **41**:549–562.

Morino, S., H., Imataka, Y. V., Svitkin, T. V., Pestova, and N. Sonenberg. 2000. Eukaryotic translation initiation factor 4E (eIF4E) binding site and the middle one-third of eIF4G1 constitute the core domain for cap-dependent translation, and the C-terminal one-third functions as a modulatory region. *Mol Cell Biol.* **20**: 468-77.

Newman, J. F., B. Cartwright, T. R. Doel, and F. Brown. 1979. Purification and identification of the RNA-dependent RNA polymerase of foot-and mouth disease virus. *J. Gen. Virol.* **45**:497–507.

Niedzwiecka, A., J., Marcotrigiano, J., Stepinski, M., Jankowska-Anyszka, A., Wyslouch-Cieszyńska, M., Dadlez, A., Gingras, P., Mak, E., Darzynkiewicz, N., Sonenberg, S. K. Burley, and R., Stolarski. 2002. Biophysical studies of eIF4E cap-binding protein: recognition of mRNA 5' cap structure and synthetic fragments of eIF4G and 4E-BP1 proteins. *J Mol Biol.* **319**:615-625.

Niepmann, M., A., Petersen, K., Meyer, and E. Beck. 1997. Functional Involvement of Polypyrimidine Tract-Binding Protein in Translation Initiation Complexes with the Internal Ribosome Entry Site of Foot-and-Mouth Disease Virus. *J. Virol.* **71**:8330-8339.

Niepmann, M. Internal translation initiation of picornaviruses and hepatitis C virus. 2009. *Biochim Biophys Acta*, **1789**: 529–541.

Novak, J. E., and K. Kirkegaard. 1991. Improved method for detecting poliovirus negative strands used to demonstrate specificity of positive strand encapsidation and the ratio of positive to negative strands in infected cells. *J. Virol.* **65**:3384–3387.

Nugent, C.I., and K. Kirkegaard. 1995. RNA binding properties of poliovirus subiral particles. *J. Virol.* **69**:13-22.

Ohlmann, T., V. M., Pain, W., Wood, M., Rau, and S. J., Morley. 1997. The proteolytic cleavage of eukaryotic initiation factor (eIF) 4G is prevented by eIF4E binding protein (PHAS-I; 4E-BP1) in the reticulocyte lysate. *EMBO J* **16**:844–855.

Pain, V. M. 1996. Initiation of protein synthesis in eukaryotic cells. *Eur J Biochem*, **236**:747-771.

Palmenberg, A. C. 1990. Proteolytic processing of picornaviral polyprotein. *Annu. Rev. Microbiol.* **44**:603–623.

Paul, A. V., A., Schultz, S. E., Pincus, S., Oroszlan, and E. Wimmer. 1987. Capsid protein VP4 of poliovirus is N-myristoylated. **84**: 7827-31.

Paul, A. V. 2002. Possible unifying mechanism of picornavirus genome replication, p. 227–246. *In* B. L. Semler and E. Wimmer (ed.), *Molecular biology of picornaviruses*. ASM Press, Washington, D.C.

Paul, A. V., J., Yin, J., Mugavero, E., Rieder, Y., Liu, and E. Wimmer. 2003. A “slideback” mechanism for the initiation of protein-primed RNA synthesis by the RNA polymerase of poliovirus. *J Biol Chem* **278**:43951-43960.

Pfaff, E., H. J. Thiel, E. Beck, K. Strohmaier, and H. Schaller. 1988. Analysis of neutralizing epitopes on foot-and-mouth disease virus. *J. Virol.* **62**:2033–2040.

Piccone, M. E., E. Rieder, P. W. Mason, and M. J. Grubman. 1995. The foot-and-mouth disease virus leader proteinase gene is not required for viral replication. *J. Virol.* **69**:5376–5382.

Pierschbacher, M. D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**:30–33.

Pilipenko, E. V., S. V. Maslova, A. N. Sinyakov, and V. I. Agol. 1992a. Towards identification of cis-acting elements involved in the replication of enterovirus and rhinovirus RNAs: a proposal for the existence of tRNA-like terminal structures. *Nucleic Acids Res.* **20**:1739–1745

Pilipenko, E. V., A. P. Gmyl, S. V. Maslova, Y. V. Svitkin, A. N. Sinyakov, and V. I. Agol. 1992b. Prokaryotic-like cis elements in the cap-independent internal initiation of translation on picornavirus RNA. *Cell* **68**:119–131.

Pilipenko, E. V., K. V. Poperechny, S. V. Maslova, W. J. Melchers, H. J. Slot, and V. I. Agol. 1996. Cis-element, oriR, involved in the initiation of (-) strand poliovirus RNA: a quasi-globular multi-domain RNA structure maintained by tertiary (‘kissing’) interactions. *EMBO J.* **15**:5428–5436.

Ptushkina M., T., von der Haar, M. M., Kari, J. M., Hughes, and J. E., McCarthy. 1999. Repressor binding to a dorsal regulatory site traps human eIF4E in a high cap-affinity state. *EMBO J* **18**:4068–4075.

Pyronnet, S., Imataka, H., Gingras, A. C., Fukunaga, R., Hunter, T. and N. Sonenberg. 1999. Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. *Embo J*, **18**:270-279.

Rieder, E., T. Bunch, F. Brown, and P. W. Mason. 1993. Genetically engineered foot-and-mouth disease viruses with poly(C) tracts of two nucleotides are virulent in mice. *J. Virol.* **67**:5139–5145.

Rieder, E., A. V. Paul, D. W. Kim, J. H. van Boom, and E. Wimmer. 2000. Genetic and biochemical studies of poliovirus *cis*-acting replication element *cre* in relation to VPg uridylylation. *J. Virol.* **74**:10371–10380.

Rivera, V. M., J. D. Welsh, and J. V. Maizel, Jr. 1988. Comparative sequence analysis of the 5' noncoding region of the enteroviruses and rhinoviruses. *Virology* **165**:42–50.

Robertson, B. H., D. M. Moore, M. J. Grubman, and D. G. Kleid. 1983. Identification of an exposed region of the immunogenic capsid polypeptide VP1 on foot-and-mouth disease virus. *J. Virol.* **46**:311–316.

Rohll, J. B., D. H. Moon, D. J. Evans, and J. W. Almond. 1995. The 3' untranslated region of picornavirus RNA: features required for efficient genome replication. *J. Virol.* **69**:7835–7844.

Rossmann, M. G., E., Arnold, J. W., Erickson, E. A., Frankenberger, J. P., Griffith, H. J., Hecht, J. E., Johnson, G., Kamer, M., Luo, and A. G. Mosser. 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. *Nature*, **317**: 145-53.

Rotbart, H. A. 1995. Human enterovirus infections. ASM Press, Washington, D. D.

Rueckert, R. R., and E. Wimmer. 1984. Systematic nomenclature of picornavirus proteins. *J. Virol.* **50**:957–959.

Santos, J., I., Gouvea, W., Judice, M., Izidoro, F., Alves, R., Melo, M., Juliano, T., Skern, and L. Juliano. 2009. Hydrolytic Properties and Substrate Specificity of the Foot and Mouth Disease Leader Protease. *Biochemistry*.

Scheper, G.C., B., van Kollenburg, J., Hu, Y., Luo, D. J. Goss, and C. G. Proud. 2002. Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. *J Biol Chem*, **277**: 3303-3309.

Schneider, R., V. I., Agol, R., Andino, F., Bayard, D. R., Cavener, S. A., Chappell, J. J. Chen, J. L. Darlix, A., Dasgupta, O., Donzé, R., Duncan, O., Elroy-Stein, P. J., Farabaugh, W., Filipowicz, M. Jr., Gale, L., Gehrke, E., Goldman, Y., Groner, J. B., Harford, M., Hatzoglou, B., He, C. U., Hellen, M. W., Hentze, J., Hershey, P., Hershey, T., Hohn, M., Holcik, C. P., Hunter, K., Igarashi, R., Jackson, R., Jagus, L. S., Jefferson, B., Joshi, R. Kaempfer, M., Katze, R. J., Kaufman, M., Kildejian, S. R., Kimball, A., Kimchi, K., Kirkegaard, A. E., Koromilas, R. M., Krug, V., Kruys, B. J., Lamphear, S., Lemon, R. E., Lloyd, L. E., Maquat, E., Martínez-Salas, M. B., Mathews, V. P., Mauro, S., Miyamoto, I., Mohr, D. R., Morris, E. G., Moss, N., Nakashima, A. Palmenberg, N. T. Parkin, T., Pe'ery, J., Pelletier, S., Peltz, T. V., Pestova, E. V., Pilipenko, A. C., Prats, V., Racaniello, G. S., Read, R. E., Rhoads, J. D., Richter, R., Rivera-Pomar, A., Sachs, P., Sarnow, G. C., Scheper, L., Schiff, D. R., Schoenberg, B. L. Semler, A., Siddiqui, T., Skern, N., Sonenberg, W., Sossin, N., Standart, S. M., Tahara, A. A., Thomas, J. J., Toulmé, J., Wilusz, E., Wimmer, G., Witherell, and M. Wormington. 2001. New ways of initiating translation in eukaryotes. *Mol Cell Biol.* **21**:8238-46.

Semler, B. L., and E. Wimmer. 2002. Molecular Biology of Picornaviruses. American Society for Microbiology, Washington, DC

Serrano, P., M. R., Pulido, M., Sáiz, and E. Martínez-Salas. 2006. The 3' end of the foot-and-mouth disease virus genome establishes two distinct long-range RNA–RNA interactions with the 5' end region. *J Gen Virol.* **87**:3013-3022.

Slepenkov, S. V., N. L., Korneeva, and R. E. Rhoads. 2008. Kinetic mechanism for assembly of the m7GpppG.eIF4E.eIF4G complex. *J Biol Chem.*, **283**: 25227-37.

Sonenberg N., and A. C., Gingras. 1998. The mRNA 5' cap-binding protein eIF4E and control of cell growth. *Curr Opin Cell Biol* **10**:268–275.

Steil, B. P., and D. J. Barton. 2009. Cis-active RNA elements (CREs) and picornavirus RNA replication. *Virus Res.*, **139**: 240-52.

Strebel, K., and E. Beck. 1986. A second protease of foot-and-mouth disease virus. *J. Virol.* **58**:893–899.

Svitkin, Y. V., L. P., Ovchinnikov, G., Dreyfuss, and N., Sonenberg. 1996. General RNA binding proteins render translation cap dependent. *Embo J*, **15**:7147-7155.

Tahara, S.M., M. A., Morgan, and A. J. Shatkin. 1981. Two forms of purified m7G-cap binding protein with different effects on capped mRNA translation in extracts of uninfected and poliovirus-infected HeLa cells. *J. Biol. Chem.* **256**: 7691–7694.

Tamkun, J. W., D. W. DeSimone, D. Fonda, R. S. Patel, C. Buck, A. F. Horwitz, and R. O. Hynes. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell* **46**:271–282.

Tomoo, K., X., Shen, K., Okabe, Y., Nozoe, S., Fukuhara, S., Morino, T., Ishida, T., Taniguchi, H., Hasegawa, A., Terashima, M., Sasaki, Y., Katsuya, K., Kitamura, H., Miyoshi, M., Ishikawa, and K. Miura. 2002. Crystal structures of 7-methylguanosine 5'-triphosphate (m(7)GTP)- and P(1)-7-methylguanosine-P(3)-adenosine-5', 5'-triphosphate (m(7)GpppA)-bound human full-length eukaryotic initiation factor 4E: biological importance of the C-terminal flexible region. *Biochem J*, **362**: 539-544.

Tomoo, K., X., Shen, K., Okabe, Y., Nozoe, S., Fukuhara¹, S., Morino, M., Sasaki, T., Taniguchi, H., Miyagawa, K., Kitamura, K., Miura, and T., Ishida. 2003. Structural features of human initiation factor 4E, studied by X-ray crystal analyses and molecular dynamics simulations. *J. Mol. Biol.* **328**:365–383.

Vagnozzi, A., D. A., Stein, P. L., Iversen, and E. Rieder. 2007. Inhibition of Foot-and-Mouth Disease Virus Infections in Cell Cultures with Antisense Morpholino Oligomers. *J Virol*, **81**: 11669-11680

Volpon, L., M. J., Osborne, I., Topisirovic, N., Siddiqui, and K. L. B., Borden. 2006. Cap-free structure of eIF4E suggests a basis for conformational regulation by its ligands. *Embo J*, **25**:5138-5149.

von der Haar, T., P. D., Ball, and J. E. G. McCarthy. 2000. Stabilization of Eukaryotic Initiation Factor 4E Binding to the mRNA 5'-Cap Domains of eIF4E. *J Biol Chem.* **275**:30551-30555.

Wong, S. M., D. C., Koh, and D., Liu. 2008. Identification of plant virus IRES. *Methods Mol Biol*, **451**:125–133.

Wyckoff, E. E., J. W., Hershey, and E. Ehrenfeld. 1990. Eukaryotic initiation factor 3 is required for poliovirus 2A protease-induced cleavage of the p220 component of eukaryotic initiation factor 4F. *Proc Natl Acad Sci, U S A.* **24**: 9529-33.

Wyckoff, E. E., R. E., Lloyd, and E. Ehrenfeld. 1992. Relationship of eukaryotic initiation factor 3 to poliovirus-induced p220 cleavage activity. *J Virol.*, **66**: 2943-51.

Zapata, M. J., Martínez, M. A., and J. M. Sierra. 1993. Purification and Characterization of Eukaryotic Polypeptide Chain Initiation Factor 4F of *Drosophila melanogaster* Embryos. *J Biol Chem.* **269**:18047-52.

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1996-2002	Secondary school (BG und BRG) in Gänserndorf
2002-2010	Student of chemistry at the University of Vienna
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