Evasion of innate antiviral responses by picornavirus security proteins

Linda Jessica Visser

**Evasion of innate antiviral responses by picornavirus
security proteins**

**Het ontwijken van aangeboren antivirale responsen door picornavirus
security proteins**

(met een samenvatting in het Nederlands)

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Linda Jessica Visser

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Promotor:

Prof. dr. F.J.M. van Kuppeveld

Copromotoren:

Dr. M.A. Langereis

Dr. R.J. de Groot

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Chapter 1

General Introduction

The family *Picornaviridae* encompasses several notorious human and animal pathogens including poliovirus (genus *Enterovirus*), hepatitis A virus (genus *Hepatovirus*) and foot-and-mouth disease virus (genus *Aphthovirus*). In 1898, foot-and-mouth disease virus was the first 'infectious agent smaller than a bacteria that could transmit disease amongst animals' that was discovered (1). Shortly after, in 1908, poliovirus was the first identified virus that transmits disease in humans (2). Although these pathogens were called viruses, the Latin term for poison, it was largely unknown what these pathogens were or how they transmitted disease. Since their discovery, these two viruses have been studied extensively and had a vital role in the development of the field of molecular virology. Understanding how these viruses translate and replicate their genome, as well as how they manipulate cellular processes, has also contributed to our current understanding of the molecular processes of the cell.

Replication of picornaviruses, like for all other viruses, can be recognized by their hosts and this triggers the activation of antiviral responses. Viruses must counteract these antiviral responses in order to spread to neighboring tissues and successfully establish infection. In this thesis, we study how selected picornaviruses evade innate antiviral responses. This chapter describes the most important picornavirus genera including their pathogenesis, the general picornavirus life cycle, antiviral responses that are relevant to the subsequent chapters, and the current knowledge of the mechanisms used by picornaviruses to evade these antiviral responses.

I. Picornavirus classification and pathogenesis

Picornaviruses are small non-enveloped viruses that carry a positive-strand RNA genome of ~7-8 kb, which categorizes them in Baltimore class IV. The picornavirus family traditionally consisted of 9-12 genera (3) and the viruses belonging to these genera have been studied most intensively. The discovery of novel picornaviruses in recent years has led to a rapid expansion of the family, which currently consists of 47 genera and 110 species (Fig 1)(4). Unfortunately, many of these novel picornaviruses remain poorly characterized and the majority of our understanding of picornavirus biology comes from the viruses belonging to the traditional genera. These genera include the picornaviruses that cause clinical disease, both in humans and in animals, while most newly discovered viruses appear to cause asymptomatic infection.

Most picornaviruses transmit via the fecal-oral route, while some picornaviruses can also transmit via aerosols or direct contact (4). Upon an initial round of replication at the primary site of infection, often within the digestive tract, some picornaviruses can

disseminate to other organs. For example, poliovirus initially replicates in the oropharynx and/or the digestive tract, and subsequently has the potential to spread to the central nervous system (2). Viral replication in the neurons can result in poliomyelitis, which affects 1-2% of poliovirus-infected individuals (2). Poliomyelitis is an acute flaccid paralysis disease that results in life-long disabilities and occasionally death. Here, we introduce the genera that cause clinical disease and/or that are of importance for the subsequent chapters of the thesis.

1. Enteroviruses

The genus *Enterovirus* is the largest genus amongst the family, encompassing 15 species of which 7 contain human pathogens (4). Viruses amongst the enterovirus A-D species and rhinovirus A-C species can cause disease in humans. Poliovirus, the best-known enterovirus, is a member of the enterovirus C species. Coxsackieviruses, echoviruses and numbered enteroviruses can induce a number of symptoms including viral meningitis, pericarditis, myocarditis and rare cases of acute flaccid paralysis (5).

Several enteroviruses have gained attention over recent years as potential health threats. One of these is EV-A71, the causative agent of hand-foot-and-mouth disease (HFMD). HFMD is characterized by fever accompanied by blisters on the hands and feet, and in the oral cavity. HFMD often affects young children. Disease progression is usually mild, but severe complications such as encephalitis, meningitis and acute flaccid paralysis can occur. Severe outbreaks of EV-A71 in Asia in recent years were associated with many cases of HFMD, neurological complications and fatalities (6, 7). This led to the development of an EV-A71 vaccine, which is currently only commercially available in China (8). A second enterovirus that has recently gained attention is EV-D68. This enterovirus normally replicates in the respiratory tract and induces mild symptoms. In recent years, the virus has been increasingly detected in hospitalized children and recognized to cause symptoms such as severe bronchiolitis or pneumonia (9). Large EV-D68 outbreaks in the USA in 2014, 2016 and 2018 coincided with an increase in cases of acute flaccid myelitis, a form of acute flaccid paralysis. Although EV-D68 was only sporadically detected in the cerebrospinal fluid of AFM patients, the same is true for known neurotropic viruses, including poliovirus and EV-A71 (9, 10). A significant increase in antibodies directed to non-polio enteroviruses, predominantly directed to EV-D68 but also EV-A71, could be identified in the CSF of the recent AFM patients (11), demonstrating the potential of these enteroviruses to induce polio-like disease.

Figure 1: The picornaviridae family. P1 protein phylogenetic tree showing the relationships between the members of the family *Picornaviridae* as described in the 2017 ICTV report (4). Unfortunately, the freely available data file has not been updated for the 2019 ICTV report and therefore this tree only contains 35 genera. Genera studied in this thesis are highlighted in orange. Data file for the maximum likelihood tree was downloaded from the ICTV website. The original datafile was produced with MrBayes using the aamodel (blosum) and the tree was rooted to the ampivirus outgroup. This visualization was made with FigTree.

The rhinoviruses are classified as three separate species within the genus *Enterovirus* and replicate in the respiratory tract. Typically, they induce the common cold, the most common human viral disease with a significant socioeconomic burden due to missed working days and visits to healthcare practitioners (12, 13). In fact, the total socioeconomic impact of all non-influenza viral respiratory infections in the United States accumulates to \$ 40 billion, annually (14). Additionally, rhinovirus infections can have severe complications and contribute to exacerbations of asthma and COPD. Especially rhinovirus A and C infections can cause severe disease in young children, such as bronchiolitis and wheezing, which may require admission of pediatric patients to the intensive care unit (12, 13).

2. Other human picornaviruses

Other clinically relevant picornaviruses that infect humans can be found amongst the *Parechovirus*, *Kobuvirus*, and *Hepatovirus* genera and these genera will be discussed briefly.

Infection with a human parechovirus usually leads to mild respiratory or gastrointestinal symptoms, similarly to enterovirus infection. However, parechovirus infection can also lead to severe complications such as neonatal sepsis or sudden infant death (15). Particularly human parechovirus 3 has the potential to induce meningoencephalitis, which can be life-threatening (16).

Aichivirus, the prototypic member of the kobuviruses, was first identified in 1989. People experienced gastrointestinal symptoms after consuming raw oysters, which were contaminated with an unidentified virus. The seroprevalence of aichivirus is high (80-95 % in adults), indicating that this virus is wide-spread in the human population and may indicate that subclinical infections is common (17).

Another important human pathogen is hepatitis A virus (HAV), a member of the hepatoviruses. HAV causes acute liver disease that is usually self-limiting, but may occasionally lead to complications requiring liver transplantation, and can be fatal. A vaccine for HAV is available, but unfortunately this virus remains endemic in most countries (18).

3. Aphthoviruses

The genus *Aphthovirus* exclusively contains veterinary pathogens and is best known for foot-and-mouth-disease virus (FMDV). FMDV causes blisters amongst the feet, mouth and mammary glands of cloven-hoofed animals (i.a. livestock animals). The virus is highly contagious. Therefore, outbreaks are met with extensive quarantine measures and international livestock trade restrictions. The virus has been eliminated from most developed countries, but unfortunately remains endemic in most of Africa and Asia, regions that economically depend predominantly on agriculture (19). The socioeconomic consequences of FMDV infections can be divided into direct effects (e.g. loss of animals and delay in the sales of animal products) and indirect effects (e.g. costs of vaccination and diagnostic testing). In endemic regions, these costs accumulate to \$ 6.5-21 billion, annually (20). The genus also encompasses the equine rhinitis A and B viruses (ERAV and ERBV), but these infections are mild and much less contagious.

4. Cardioviruses

Cardioviruses primarily circulate amongst rodents, although they can also infect other animals. Cardioviruses can be divided into two species; the cardiovirus A species consists of encephalomyocarditis virus, while the cardiovirus B species encompasses Theiler's murine encephalitis virus, Saffold virus and Vilyuisk human encephalitis virus.

Encephalomyocarditis virus (EMCV) circulates amongst rodents worldwide. This virus can also infect captive animals such as pigs, elephants and non-human primates. In these animals the virus causes encephalitis and myocarditis, and infections have a high fatality rate. EMCV infection in humans has been reported but appears to be asymptomatic or associated with very mild symptoms (21). Another prime example of a rodent cardiovirus is Theiler's murine encephalitis virus (TMEV). TMEV infection normally presents as mild gastroenteritis, but infections of the central nervous system also occur. Experimental TMEV infection leads to chronic demyelination of the nerves and causes hind limb paralysis. The lesions are reminiscent of those found in multiple sclerosis patients, and TMEV infection is frequently used as a model system to study neuron demyelination (22).

Saffold virus (SAFV) is a human-tropic cardiovirus that was first discovered in 2007. The virus is most frequently associated with respiratory and gastrointestinal symptoms, but complications in the endocrine, cardiovascular and neurological systems as a result of SAFV infection have also been observed (23). It had been suggested that another cardiovirus also affects humans. A small isolated population of indigenous people around the Vilyui river in Russia has historically suffered from encephalitis, and this has been attributed to infection with Vilyuisk human encephalitis virus (VHEV) (24). However, the cerebrospinal fluid (CSF) of patients was injected in mice, before isolating the virus. As a result, it is unclear whether VHEV originates from man or mice. The species-specificity of one of the viral proteins (L*) unambiguously demonstrated that VHEV has a murine origin, suggesting that VHEV is a contaminant murine virus rather than a human cardiovirus (25).

II. Picornavirus genome organization and life cycle

Viruses are obligate intracellular pathogens that rely on the host metabolism to replicate. The picornavirus life cycle, from virus entry to release of new progeny, is depicted in figure 2 and described below. This section aims to describe the life cycle of all picornaviruses; however, the research on this topic has focused on only a few genera. Where dogmas are genus-specific, this will be acknowledged.

Infection starts with binding to the viral receptor to induce receptor mediated endocytosis (26). Picornaviruses use a variety of cell-surface protein as receptors, the exact receptor varies both between and within genera. Enteroviruses often use a member of the Ig-like superfamily or an integrin to enter the cell (27). Meanwhile, FMDV can bind to at least four different integrins, but can also use heparan sulfate as a receptor (28). In the endosome, the virus is uncoated to release its genome from the virion into the cytoplasm. This occurs via a virus-induced pore in the endosomal membrane.

Once in the cytoplasm, the viral genome is directly translated. Contrary to host mRNAs, the viral genome lacks a 5' cap structure, which is normally used to initiate ribosome assembly. Instead, ribosomes are recruited to the viral genome via an RNA structure in the 5' UTR of the genome, known as the Internal Ribosomal Entry Site (IRES) (29, 30). IRES-mediated translation initiation requires some, but not all, translation initiation factors that direct normal cap-dependent translation. Which translation initiation factors are required depends on the exact IRES. Picornavirus IRESs have been sorted into 5 classes and these are distributed across the different genera (reviewed in (31)).

Figure 2: The picornavirus life cycle. This figure was made with BioRender.

The viral RNA is directly translated as one polyprotein that encompasses the capsid proteins (VP4, VP2, VP3, VP1; collectively also known as P1) and the nonstructural proteins (2A-C and 3A-D), which are needed to replicate the viral genome. In some genera, an additional protein, the leader (L), is encoded at the start of the polyprotein. The polyprotein is subsequently processed to 11 or 12 viral proteins and several stable intermediates (26). The majority of the polyprotein cleavages are attributed to the main viral protease, 3C^{pro}. Some genera encode additional proteases, which contribute to the cleavage of the viral polyprotein. Notable examples are the L^{pro} of aphthoviruses and the 2A^{pro} of enteroviruses. Aphthovirus L^{pro} is encoded as the first protein of the polyprotein and cleaves at its own C-terminus to release itself from the polyprotein. Enterovirus 2A^{pro} cleaves at its N-terminus to release the nonstructural proteins from the capsid encoding region. The viral proteases also cleave several host factors to remodel the cellular landscape to benefit viral replication and/or evade the immune system. The latter will be discussed in more detail in the section “Evasion of innate antiviral responses by picornaviruses”.

The first step in the replication of the viral genome is the generation of an RNA strand of negative polarity ((-)-strand synthesis) by the viral RNA-dependent RNA polymerase (3D^{pol}), resulting in a dsRNA replication intermediate. The negative strand subsequently serves as a template for synthesis of multiple new genomes of positive polarity ((+)-strand synthesis). These new viral genomes can either enter another round of translation and replication, or can be encapsidated to form new virions (26). Viral genome replication takes place at virus-induced membranous structures, termed replication organelles (ROs) (26, 32). ROs are newly formed organelles that are generated using host membrane components derived from several organelles, which varies between genera (32). Most knowledge of ROs is based on enterovirus replication, although some studies on cardioviruses are available. While we aim to describe picornavirus ROs, a certain level of bias towards enterovirus and cardiovirus replication is unavoidable. Cardiovirus ROs are likely generated with host membrane components derived from the ER (33, 34), while enteroviruses utilize membrane components from the ER, ERGIC and the Golgi apparatus (35–39). The structure of ROs is dynamic and changes as infection progresses. Early in infection predominantly single membrane vesicles and tubules are formed, and these transform into double membrane vesicles (DMVs) as infection progresses (34, 37, 40, 41). A role for the autophagy machinery in the formation of ROs and/or the changes in RO structure has been suggested (35, 36, 39, 42). ROs are believed to support replication by scaffolding the molecules involved in RNA replication (43). It has also been suggested that the membranous structures shield the viral genome and its replication intermediate, dsRNA, from recognition by the innate immune system (44), although this was not observed in another study (45).

The newly formed capsid proteins, together with nascent viral RNA, assemble into full virions. In the case of enteroviruses, virions are assembled with VP0, the precursor of VP2 and VP4. Assembly of the virion and the interaction between VP0 and the viral genome induces the processing of VP0 into VP2 and VP4, to mature the virion (26). Textbooks describe picornaviruses as obligate lytic viruses, meaning that they break out of the dying cell at the end of the replication cycle. However, evidence is accumulating that new virions are released in a non-lytic manner at earlier timepoints, via fusion of virus-containing DMVs or autophagosomes with the plasma membrane (42, 46, 47).

III. Innate antiviral responses

Viral infections are recognized by the host's immune system. Broadly, the immune system consists of two branches; the innate and the adaptive immune system. The well-known T-cells, B-cells, and antibodies all belong to the adaptive immune system and are essential for the eventual clearing of infections. However, raising an adaptive immune response may take several days and requires the body to first identify the pathogen. This is the role of the innate immune system. The innate immune system is trained to discriminate ‘self’ from ‘non-self’, and acts as a first line of defense. In order to identify ‘non-self’ molecules, the innate immune system uses an extensive repertoire of pattern recognition receptors (PRRs) to identify pathogen associated molecular patterns (PAMPs) (48). Well-known examples of PAMPs are bacterial macromolecules such as lipopolysaccharide or flagellin. The innate immune system consists of some dedicated sentinel cells (i.a. macrophages, dendritic cells and NK cells). These cells predominantly depend on Toll-like receptors (TLRs) to detect pathogens (48). However, a fundamental aspect of the innate immune system is the ability of all nucleated cells to produce type I interferons, a fundamental pro-inflammatory cytokine that is at the base of raising an innate immune response.

1. Type I interferon

All nucleated cells can detect intracellular PAMPs via RIG-I-like receptors (RLRs). These sensors subsequently initiate a signaling cascade to induce the production of type I interferons. The RLRs are a family of DExH/D box helicases that consists of RIG-I, MDA5 and LGP2. Both RIG-I and MDA5 have caspase activation and recruitment domains (CARD domains) that are essential for initiating the signaling cascade. LGP2 lacks a CARD domain and thereby cannot signal to downstream molecules, instead LGP2 regulates both RIG-I and MDA5 (48). MDA5 and RIG-I recognize infection by different groups of viruses. Some viruses are detected by one sensor (e.g. influenza A is recognized by RIG-I (49)), while others are detected by both sensors (e.g. measles virus)(50, 51). To explain these differences, many studies have focused on the molecular characteristics of RIG-I and MDA5 ligands. RIG-I

recognizes RNA molecules that contain short stretches of dsRNA (at least 20 bp) and a 5' triphosphate group, while MDA5 recognizes long dsRNA molecules (> 1kb) (48, 52). The replication of the picornavirus genome generates long dsRNA replication intermediates, which have been shown to activate MDA5 (53). Nevertheless, it was recently suggested that RIG-I may also be involved in the recognition of enterovirus infection in primary cells (54).

Upon ligand recognition, the CARD domain of MDA5/RIG-I interacts with the CARD domain of mitochondrial antiviral signaling protein (MAVS, also known as IPS-1, VISA or Cardif). MAVS then activates several different TNF receptor-associated factors (TRAF proteins) to activate both the TBK1/IKK- ϵ signaling pathway and the IKK pathway. TRAF2/3 homo- and heterodimers activate TBK1, a kinase that phosphorylates the transcription factors interferon regulatory factor 3 (IRF3) and IRF7. Phosphorylation of IRF3 and IRF7 allows these transcription factors to homodimerize and translocate to the nucleus. Simultaneously, TRAF3/6 homo- and heterodimers activate the IKK- $\alpha/\beta/\gamma$ complex, which phosphorylates the I κ B α complex. In its unphosphorylated form, the I κ B α complex binds to the transcription factor NF- κ B to inhibit its translocation to the nucleus. In the nucleus IRF3, IRF7 and NF- κ B induce the transcription of IFN- α/β and other proinflammatory cytokines (Figure 3).

The RLR signaling cascade needs to be tightly regulated to prevent tissue damage resulting from an excessive inflammatory response. Therefore, the pathway relies heavily on post translational modifications (PTMs) of signaling molecules. Addition or removal of PTMs is a much quicker biochemical reaction than the relatively slow process of protein synthesis or degradation. Besides phosphorylation of signaling molecules (described above), the pathway is regulated by ubiquitination (reviewed in (55, 56) and depicted in Figure 3). Ubiquitination is the addition of ubiquitin (an 8 kDa protein) to surface-exposed lysines via an iso-peptide bond. Ubiquitin itself can also be ubiquitinated, resulting in the formation of ubiquitin chains. These chains have varying 3D structures, depending on the lysine that is used to form the chain. Of importance for the RLR signaling cascade are K-48 and K-63 linked ubiquitin chains, which signal for protein degradation (K-48) or positively influence signal transduction (K-63) (55, 56). The K-63 linked ubiquitination of RIG-I is well-characterized. Upon RNA binding and RIG-I activation, the N-terminal CARD domain of RIG-I is first dephosphorylated, before being ubiquitinated (57, 58). The presence of this ubiquitin chain stabilizes its interaction with MAVS (57). Intriguingly, MDA5 appears to be regulated via phosphorylation and dephosphorylation, rather than ubiquitination (58). MAVS itself is also regulated by K-63 ubiquitination, which promotes the recruitment of IKK- ϵ and the activation of IRF3 (59). Downstream of MAVS, ubiquitination plays an important role in both signaling arms; the signaling cascade leading to NF- κ B activation and the one leading to activation of IRF3 and IRF7. Upon interaction with upstream signaling

molecules, TRAF proteins auto-ubiquitinate and this allows their respective downstream signaling complexes to bind (60–64). TBK1 is also ubiquitinated, which is essential for its enzymatic activity and thus for the activation of IRF3 and IRF7 (65, 66). Meanwhile, K-48 ubiquitination and subsequent proteasomal degradation of RIG-I, MDA5, MAVS, TRAF3, TRAF6, TBK1 and the transcription factors IRF3, IRF7 and NF- κ B, downregulates the inflammatory response (67–74).

Figure 3: RLR signaling induces expression of type I interferons. This figure was made with BioRender.

IFN- α/β is a cytokine that upon its secretion binds the interferon receptor (IFNAR), both on the infected cell and on uninfected neighboring cells. Upon IFN- α/β binding, Janus kinase (JAK) phosphorylates IFNAR to recruit the signal transducer and activator of transcription (STAT) proteins. The STAT proteins themselves are also phosphorylated by JAK, which allows them to dimerize. STAT dimers subsequently translocate to the nucleus to initiate expression of hundreds of interferon stimulated genes (ISGs), which have antiviral functions. Expression of the ISGs promotes an antiviral state and thereby protects uninfected cells from viral infection. There is a wide range of ISGs with many

different activities. This group includes the signaling proteins mentioned above (i.a. PRRs, IRFs, JAK and STATs), pro-apoptotic proteins, but also proteins with an antiviral effector function (75).

Among the first discovered ISGs were the oligoadenylate synthetases (OAS) and RNase L. OAS synthesize 2'-5' oligoadenylates upon recognition of dsRNA molecules. These oligoadenylates act as second messengers to activate the endonuclease RNase L, before being degraded by cellular 2'-phosphodiesterases, which can break 2'-5' bonds, and phosphatases, which remove the 5' phosphate group. Upon its activation, RNase L degrades cytosolic RNA molecules of both viral and cellular origin (75–78). Degradation of viral RNA is directly antiviral, while the degradation of cellular RNA molecules damages the host. The degradation of cellular RNA molecules promotes apoptosis, an effective host strategy when homeostasis cannot be restored and the cell succumbs to the infection.

In recent years, ISG15 has gained much attention of researchers. This ubiquitin-like molecule can be covalently linked to lysine residues in target proteins, similar to ubiquitin. The first studies into ISG15 showed it was largely protective against viral infection in mice (79–82). However, people with an ISG15-deficiency are not particularly sensitive to viral infection (83). Of note, several signal transduction proteins in the IFN signaling cascades (i.a. RIG-I, IRF3 and STAT1) can be ISGylated (84–87). In addition to the ISGylation of target proteins, many other functions of ISG15 have been proposed, both intracellular and extracellular (88). However, at this time there is no comprehensive understanding of ISG15's different functions and its significance to antiviral host responses.

Another key ISG is Protein kinase R (PKR). PKR is the dsRNA binding protein that initiates the cellular stress response upon viral infection, which will be discussed in more detail below.

2. The cellular stress response

Cells have four sensor proteins (PKR, PERK, GCN2 and HRI) that recognize different types of cellular stress and that activate the cellular stress response. Activation of this response halts translation and gives the cell the opportunity to restore homeostasis. PKR recognizes viral dsRNA molecules (89), while PERK recognizes ER stress (90, 91) and GCN2 recognizes malnutrition (92, 93). HRI was originally described to be activated under low iron or heme conditions (94), but can also be activated by toxic heavy metal ions, and heat shock (95, 96).

Upon binding of viral dsRNA, PKR auto-phosphorylates leading to its dimerization and activation. Activated PKR subsequently phosphorylates the α -subunit of translation initiation factor 2 (eIF2 α) to halt translation (89) (depicted in figure 4). In an unstressed cell, eIF2 is bound to GTP and an initiator tRNA. GTP-loaded eIF2, together with translation initiation factors 1, 3, 5 and the small ribosomal subunit, is known as the 43S pre-initiation complex. Simultaneously, translation initiation factors 4A, 4E and 4G (together: eIF4F) bind the cap structure of the mRNA. The 43S pre-initiation complex can recognize and bind the eIF4F complex to initiate ribosome assembly, and scans the mRNA until it reaches a functional start codon. The energy required for translation initiation is generated by the hydrolysis of the GTP that is bound to eIF2. Subsequently, eIF2-GDP is released and the ribosome continues translating the mRNA. In order to start another round of translation, the eIF2-GDP complex needs to be reloaded with GTP and an initiator tRNA. To this end, eIF2-GDP binds to its guanidine exchange factor (GEF), the eIF2B complex. eIF2B ensures the release of the GDP molecule and this allows a new GTP molecule to take its place (97). The GEF is the rate limiting factor in this reaction. The high affinity interaction between phosphorylated eIF2 and eIF2B interferes with eIF2B's GEF function (98). Thereby, the binding of p-eIF2 to eIF2B prevents the GDP-to-GTP exchange of eIF2 molecules and effectively halts translation initiation (reviewed in (99)).

Halted translation results in an accumulation of untranslated mRNA molecules in the cell. RNA that is devoid of ribosomes is recognized by a group of RNA binding proteins (i.a. G3BP1, G3BP2, TIA-1 and TIAR). Accumulation of these mRNA-RNA binding protein complexes results in the formation of aggregate structures, known as stress granules (SGs) (Figure 4). Besides mRNAs and the RNA binding proteins, SGs also contain several translation initiation factors. SG formation depends both on protein-protein interaction (between the RNA binding proteins) and RNA-RNA interactions. SGs themselves were also suggested to be antiviral. Cells devoid of PKR, and thus unable to form SGs upon viral infection, produce less IFN- α/β compared to wild-type cells upon infection (100, 101). Additionally, numerous signaling molecules (i.e. RIG-I, MDA5 and PKR) locate to SGs (100, 102, 103) and their localization to SGs was suggested to aid their signaling capacities (102, 104). The exact antiviral role of SGs remains to be established, to date, the most important argument for their antiviral role is the fact that many viruses actively prevent SG formation (105–111).

Figure 4: Activation of the cellular stress response upon viral infection. This figure was made with BioRender.

IV. Evasion of innate antiviral responses by picornaviruses

In order to establish an infection, viruses must overcome the antiviral effects of the defense mechanisms of the host. Picornavirus 2A and Leader proteins evade innate antiviral responses, and manipulate host gene expression and host translation. Because of these functions, 2A and L are also referred to as ‘security proteins’ (112). The security proteins are the most diverse picornaviral proteins, inter-genera conservation is extremely low. Still, this diverse group of proteins has evolved to evade innate antiviral responses and manipulate general host metabolism. Additionally, the 3C protease may contribute to the evasion of host defenses, by cleavage of several host factors. Understanding the underlying molecular mechanisms not only adds to our understanding of picornavirus biology, but also illuminates unknown details of innate antiviral pathways.

1. Inhibition of host translation

Perhaps best studied is the effect of the different security proteins on translation of capped host mRNAs. Interfering with host translation can reduce the expression of antiviral gene products (e.g. IFN- α/β and ISGs), and thus be beneficial for the virus. Enteroviruses and FMDV are known to rapidly shut down cap dependent host translation, resulting in the predominant synthesis of viral proteins (113–115). 2A^{pro} of enteroviruses and L^{pro} of aphthoviruses (i.a. FMDV) cleave translation initiation factor 4G (eIF4G) (116–118), which prevents the recruitment of pre-initiation complexes to the 5' cap-structure that is present on host mRNAs. The viral genome does not have a cap and thus its translation is not inhibited by eIF4G cleavage. Instead translation initiation factors are recruited to the IRES structure, present in the viral genome. The cleavage of eIF4G was shown to positively influence IRES-mediated translation (119–121). 2A^{pro} of enteroviruses also targets poly(A)-binding protein (PABP), which is involved in circularization of the translating mRNA, thereby enhancing translation of capped host mRNAs (122, 123).

Security proteins that lack proteolytic activity can also influence host translation, the prime examples are the security proteins of cardioviruses. Infection with wildtype EMCV negatively affects host translation (124). Cardiovirus 2A localizes to the nucleolus (125), interacts with ribosomes/ribosomal RNA (126, 127), and directs translation from cap-dependent translation to IRES-dependent translation (127). These collective observations suggest that 2A is responsible for mediating shut-off of host translation. Cardiovirus L was also suggested to inhibit translation (128), but no mechanism has been elucidated. Cardiovirus 2A and L interact with each other and this interaction is important for the localization and function of both 2A and L (129). This may explain why L, indirectly, affects cap-dependent translation.

2. Inhibition of nucleocytoplasmic trafficking and transcription

Cardioviruses disrupt nucleocytoplasmic trafficking via their L protein (130–133). L interacts with RAN-GTPase, a protein that regulates nuclear transport by directing the activity of nuclear cargo transporters (e.g. importins) (131). Upon cardiovirus infection, specific nuclear pore proteins (nucleoporins, or NUPs) are hyper phosphorylated (133, 134). The resulting negative charge on these proteins induces the disruption of the nuclear pore complex, thereby interfering with nucleocytoplasmic trafficking and simultaneously facilitating passive diffusion. Via its interaction with RAN, L most likely localizes to the nuclear pores, where it directs a kinase to phosphorylate the NUPs. Enteroviruses also disrupt nucleocytoplasmic trafficking, and this has been attributed to 2A^{pro}. 2A^{pro} of multiple enteroviruses cleaves NUPs (135–140), resulting in the collapse of the nuclear pore complex and inhibition of active nuclear transport.

In addition to interfering with nuclear transport, picornaviruses affect host transcription. Poliovirus 3C^{pro} and its precursor 3CD can inhibit RNA polymerase I, II and III (141–143) by cleaving many different transcription factors, including well-known transcription factors such as TATA-binding protein (142, 144, 145) and p53 (146) (reviewed in (147)). The effects of picornavirus security proteins on transcription have not been studied in great detail, but the nucleocytoplasmic trafficking disorder induced by enterovirus 2A^{pro} may be required for 3C^{pro} to enter the nucleus, thereby allowing 3C^{pro} to inhibit transcription. In addition, it is noteworthy that FMDV L^{pro}, which does not induce NCTD, accumulates in the nucleus (148, 149) and a number of nuclear proteins (i.e. Gemin5 and ADNP) have been identified as substrates and/or interaction partners of FMDV L^{pro} (150, 151).

3. Manipulation of RLR signaling and the downstream JAK-STAT pathway

Picornaviruses also interfere with both the induction of IFN- α/β (RLR signaling) and its downstream signaling (JAK-STAT pathway). Multiple security proteins, as well as 3C^{pro} of several genera, have been suggested to downregulate these signaling cascades to evade the antiviral effects of IFN- α/β .

During enterovirus infection, IFN- α/β gene transcription is actively suppressed. Several enteroviruses interrupt the RLR signaling cascade upstream of IRF3 phosphorylation and dimerization (152–155), although the exact underlying molecular mechanism remain to be elucidated. Multiple RLR signaling proteins are cleaved by the two enterovirus proteases, 2A^{pro} and 3C^{pro}. Upon overexpression of 3C^{pro}, cleavage of RIG-I, MAVS, IRF7 and IRF9 was observed (152, 155–160), while 2A^{pro} was shown to cleave MDA5 and MAVS (152, 153). However, it remains to be determined whether the cleavage events observed upon ectopic overexpression of the viral protease also occur during infection and/or contribute to the suppression of IFN- α/β gene expression. The 2A^{pro} and 3C^{pro} of enteroviruses were also suggested to inhibit the downstream JAK-STAT signaling pathway. 2A^{pro} of EV-A71 was shown to reduce the expression of the IFNAR and interfere with STAT1 and STAT2 phosphorylation (161, 162), thereby perturbing STAT activation and subsequent translocation to the nucleus. Meanwhile, 3C^{pro} of EV-A71 was shown to directly interfere with the translocation of STAT to the nucleus, without inhibition of STAT phosphorylation (163). Further insight into these mechanisms, their effects on ISG expression, and whether the effects are conserved amongst other enteroviruses, is needed.

FMDV also strongly suppresses the production of IFN- α/β , both at the mRNA and the protein level. FMDV L^{pro} is considered the dominant FMDV protein evading the induction of IFN- α/β , because a virus lacking L^{pro} (leaderless virus) fails to reduce IFN- α/β levels (164). Wildtype FMDV can almost completely block the production of IFN- α/β proteins,

but the reduction in IFN- α/β mRNA is only about ~10-fold. As described in detail in the section “Effects on host translation”, L^{pro} cleaves translation initiation factor eIF4G and this likely affects IFN- α/β protein levels. The modest reduction in IFN- α/β mRNA levels has also been attributed to the actions of L^{pro}. L^{pro} degrades the NF- κ B subunit p65 (148, 149). Furthermore, L^{pro} was shown to decrease protein levels of IRF3 and IRF7 (165). L^{pro} also possesses deubiquitinase (DUB) activity and this was suggested to suppress RLR signaling (166). The relative contribution of each of these abilities of L^{pro} on the suppression of RLR signaling remains to be determined. Intriguingly, the 3C^{pro} of FMDV was shown to cleave NEMO (also known as IKK γ) (167), and this could further dampen the induction of IFN- α/β mRNA. FMDV 3C^{pro} also prevents the translocation of activated STAT1 and STAT2 to the nucleus, thereby interfering with JAK-STAT signaling and the expression of ISGs (168).

The above may suggest that (viral) proteases are essential in circumventing RLR signaling. However, the cardioviruses depend on their leader protein, which lacks proteolytic activity, to suppress RLR signaling. Removal or mutation of the L coding region from the genomes of EMCV or Theiler's virus resulted in a tremendous increase in IFN- β mRNA levels compared to infection with the respective wt viruses (169–172). Infection with wt EMCV, but not a EMCV mutant in which L's zinc-binding domain is disrupted, was shown to prevent IRF3 phosphorylation and dimerization (152, 170), but an exact molecular mechanism remains to be identified. TMEV L also affects IRF3 activation and function to suppress IFN gene expression, although subtle differences between different strains of the virus have been reported (173). TMEV also counteracts the antiviral effects of RNase L. This virus is the only cardiovirus that expresses a 13th viral protein, named L*, which directly interacts with RNase L to inhibit its activity (174). L* is encoded in a second ORF that overlaps with L and the capsid-encoding region (P1).

4. Evasion of the cellular stress response

PKR activates the stress response upon detection of viral RNA, resulting in the phosphorylation of eIF2 and a halt in protein translation. The halted translation subsequently results in the formation of stress granules (SGs). Viruses can evade this antiviral response in two ways; either they interfere with the signaling cascade – thereby circumventing the negative effects on translation as well as SG formation – or they exclusively interfere with SG assembly.

Enterovirus 2A^{pro} cleaves translation initiation factor eIF4G. The inhibitory effect of eIF4G cleavage on host translation should theoretically trigger SG formation. Yet, poliovirus infection does not trigger massive SG formation (175), suggesting the virus counteracts this antiviral response. While IRES-translation requires eIF2 *in vitro* or during the early stages of infection, it becomes independent of eIF2 during the later stages of infection,

via a process that requires 2A^{pro} (176). Effectively, this ability makes the virus resistant to the negative effects of eIF2 α phosphorylation and non-surprisingly poliovirus infection does not inhibit the phosphorylation of PKR and eIF2 α (177). Despite the phosphorylation of eIF2 α , there are hardly any SGs observed in poliovirus infected cells (175), suggesting that the virus suppress SG formation. Several enterovirus 3C^{pro}s were shown to cleave the RNA-binding protein G3BP1, and this was suggested to negatively affect SG formation (175, 178, 179).

Cardiovirus infection does not trigger SG formation, but SGs are formed when cells were infected with cardioviruses carrying mutations in leader (103, 180). Furthermore, both EMCV and TMEV leader inhibit the phosphorylation of PKR (181, 182), suggesting that cardioviruses targets the PKR signaling pathway to prevent stress response activation.

FMDV L^{pro}, like enterovirus 2A^{pro}, stimulates eIF2-independent IRES-driven translation at later timepoints of infection (121), suggesting that FMDV infection is resistant to the antiviral effects of eIF2 α phosphorylation. While it was shown that infection with FMDV affects SG formation (183), no mechanistic insights have been provided. Exactly how FMDV suppresses the stress response remains to be elucidated.

V. Aim and outline of this thesis

This thesis aims to increase our understanding of how different picornaviruses evade the induction of type I interferons and the antiviral effects of the stress response. Chapters 2-4 focus on FMDV L^{pro} and its ability to suppress the antiviral responses. Working with FMDV requires extensive biosafety measures. To circumvent this, we inserted FMDV L^{pro} in a recombinant encephalomyocarditis virus (EMCV) to create a chimeric virus, EMCV-L^{pro}. We utilized similar chimeric EMCVs to study the evasive functions of enterovirus 2A^{pro} and 3C^{pro} in chapter 5, and the aichivirus L protein (genus *Kobuvirus*) in chapter 6.

In chapter 2 we explored whether and how FMDV evades the stress response. We demonstrate that this virus suppresses SG formation via the activity of L^{pro}. In chapter 3 we uncovered that FMDV L^{pro} has a 1000-fold higher affinity for ISG15 than for ubiquitin. Based on overexpression experiments, L^{pro}'s deubiquitinase (DUB) activity was suggested to play a role in the suppression of RLR signaling (166). However, our data suggest that L^{pro} has only very modest DUB activity. This raised the question whether L^{pro}'s deISGylase activity is involved in suppression of RLR signaling. We address this question in chapter 4, where we separated the proteolytic activity of L^{pro} towards RLR signaling proteins from its deISGylase/DUB activity via the introduction of specific mutations in L^{pro}. This allowed us to investigate which proteolytic activity is needed to reduce the induction of IFN- α/β mRNA.

Chapter 5 provides a detailed comparative analysis of the ability of 2A^{pro} and 3C^{pro} of human enteroviruses (i.e. those belonging to species A-D) to suppress both the stress response and the induction of IFN- α/β . We demonstrate that 2A^{pro}, not 3C^{pro}, is responsible for the suppression of both innate antiviral responses.

In chapter 6 we investigate whether and how kobuviruses suppress the stress response. We identify a novel interaction between aichivirus leader and translation initiation factor 2B (eIF2B), and describe how this interaction allows L to circumvent the antiviral effects of the cellular stress response.

Chapter 7 provides a brief summary of the findings, a general discussion, and directions for future research.

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Chapter 2

2

FMDV leader protease cleaves G3BP1 and G3BP2 and inhibits stress granule formation

Linda J. Visser¹, Gisselle N. Medina², Huib H. Rabouw¹, Raoul J. de Groot¹, Martijn A. Langereis¹, Teresa de los Santos², Frank J.M. van Kuppeveld^{1,#}

¹Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

²United States Department of Agriculture, Agricultural Research Service, Foreign Animal Disease Research Unit, Plum Island Animal Disease Center, Orient, NY, USA

[#]Corresponding author.

Abstract

Like other viruses, the picornavirus foot-and-mouth disease virus (FMDV, genus *Aphthovirus*), one of the most notorious pathogens in global livestock industry, needs to navigate antiviral host responses to establish an infection. There is substantial insight into how FMDV suppresses the type I IFN response, but it is largely unknown whether and how FMDV modulates the integrated stress response. Here, we show that the stress response is suppressed during FMDV infection. Using a chimeric recombinant encephalomyocarditis virus (EMCV), in which we functionally replaced the endogenous stress response antagonist by FMDV L^{pro} or 3C^{pro}, we demonstrate an essential role for L^{pro} in suppressing stress granule (SG) formation. Consistently, infection with a recombinant FMDV lacking L^{pro} resulted in SG formation. Additionally, we show that L^{pro} cleaves the known SG scaffold proteins G3BP1 and G3BP2, but not TIA-1. We demonstrate that the closely related equine rhinitis A virus (ERAV) L^{pro} also cleaves G3BP1 and G3BP2 and also suppresses SG formation, indicating that these abilities are conserved amongst aphthoviruses. Neither FMDV or ERAV L^{pro} interfered with phosphorylation of PKR or eIF2 α , indicating that L^{pro} does not affect SG formation by inhibiting the PKR-triggered signaling cascade. Taken together, our data suggest that aphthoviruses actively target scaffolding proteins G3BP1 and G3BP2 and antagonize SG formation to modulate the integrated stress response.

Importance

The picornavirus foot and mouth disease virus (FMDV) is a notorious animal pathogen that puts a major economic burden on global life stock industry. Outbreaks have significant consequences for animal health and product safety. Like many other viruses, FMDV must manipulate antiviral host responses to establish infection. Upon infection, viral dsRNA is detected which results in activation of the PKR-mediated stress response, leading to a stop in cellular and viral translation and the formation of stress granules (SG), which are thought to have antiviral properties. Here we show that FMDV can suppress SG formation via its leader protease (L^{pro}). Simultaneously we observed that L^{pro} can cleave the SG scaffolding proteins G3BP1 and G3BP2. Understanding the molecular mechanisms of FMDV's antiviral host response evasion strategies may help to develop countermeasures to control FMDV infections in the future.

Introduction

Innate antiviral responses represent the first line of defense against viral infections. Cellular antiviral signaling is initiated upon recognition of pathogen-associated molecular patterns (PAMPs) by the host. Cytosolic viral RNA can be detected by the RIG-I like receptors (RLRs). Upon recognition of viral RNA, RLRs activate signal transduction pathways resulting in the production and secretion of type I interferons (IFN- α/β) and pro-inflammatory cytokines. Secreted IFN- α/β triggers the transcription of interferon-stimulated genes (ISGs), thereby generating an antiviral state in both infected and surrounding cells that limits virus spread (1).

Besides the IFN- α/β pathway, the cellular integrated stress response (ISR) has been increasingly recognized as an antiviral pathway (2). Cells can halt translation upon different kind of cellular stress, including viral infection. Double-stranded (ds) RNA-dependent protein kinase (PKR), an ISG, detects cytosolic viral double-stranded RNA (dsRNA) and activates the stress response in virus-infected cells (3). Detection of dsRNA by PKR results in structural rearrangements in PKR leading to auto-phosphorylation. Next, activated PKR phosphorylates the alpha-subunit of eIF2. Phosphorylation of eIF2 α halts translation, thereby interfering with synthesis of viral proteins and virus propagation (3). Upon halted translation, specific nucleating factors bind specific stalled mRNA-protein complexes (mRNPs) to form cytoplasmic aggregates known as stress granules (SGs). These nucleating factors include Ras GTPase-activating protein-binding proteins 1 and 2 (G3BP1 and G3BP2), T cell-restricted intracellular antigen 1 (TIA-1) and the closely related TIAR, and multiple other factors (e.g. Caprin1 and Sam68). SGs are highly dynamic cytoplasmic aggregates. Scaffold proteins continuously cycle between the cytosol and SGs, facilitating mRNA triage, in order to direct translation towards resolving the cellular stress (2, 4).

SGs have been proposed to act as a platform for antiviral signaling (2, 4–7). The local enrichment of nucleating factors and mRNPs most likely constitutes a warning signal in the cell, recruiting numerous signaling molecules to the SGs (4). These include several signaling molecules involved in the innate antiviral response, such as RIG-I (8), MDA5 (9), TRAF2 (10) and PKR (5). Despite these observations, the importance and role of SGs as an antiviral signaling platform are still poorly understood. The most important argument that supports an antiviral role of SGs is the fact that many viruses have developed antagonistic mechanisms that interfere with SG assembly. These mechanisms include inhibition of PKR, cleavage of SG scaffold proteins by viral proteases, and sequestration of SG scaffold proteins by viral proteins (2, 11–17).

Picornaviruses, a large family of small, non-enveloped viruses that contain a single-stranded 7-8 kb RNA genome of positive polarity, actively antagonize both RLR signaling and the stress response. Viruses belonging to the genus *Enterovirus*, best known for poliovirus, actively suppress RLR signaling through their viral proteases, 2A^{pro} and 3C^{pro}, which cleave multiple proteins in the RLR signaling pathway (18–22). 2A^{pro} and 3C^{pro} also cleave signaling molecules of the IFN- α/β signaling pathway to prevent the expression of ISGs (23). Enteroviruses also actively antagonize the cellular stress response, as SGs rapidly dissolve as infection progresses. This observation has been linked to the cleavage of the SG scaffold protein G3BP1 by the viral 3C^{pro} (24), although other viral proteins have also been suggested to affect SG formation (25). Similarly, caliciviruses encode NS6, a 3C-like protease, which also cleaves G3BP1 to prevent SG assembly (11). Members of the genus *Cardiovirus* (e.g. encephalomyocarditis virus (EMCV) and Theiler's virus) encode the leader protein, one antagonist that suppresses both RLR signaling and the stress response pathway (9, 18, 26, 27). The mechanism of action of the cardiovirus leader protein, which lacks proteolytic activity, is largely unknown. Besides the leader protein, the 3C^{pro} of cardioviruses has been implicated in suppressing SG formation by cleaving G3BP1 (28), although this was not observed in another study (26).

Another well-known picornavirus is foot-and-mouth disease virus (FMDV), which is one of the most notorious pathogens in global livestock industry. FMDV infections are well known for their devastating effects on animal health and product safety and can have a huge economic impact. FMDV is a member of the genus *Aphthovirus*, which also contains bovine rhinitis A and B viruses, and equine rhinitis A virus (ERAV). Like cardioviruses, aphthoviruses encode a leader protein. FMDV leader is a papain-like protease (L^{pro}) that is best known for cleaving translation initiation factor eIF4G, thereby shutting off host mRNA translation (29). Efficient spread of FMDV infection *in vivo* relies on L^{pro} (30). Besides causing translational shutoff of host proteins, L^{pro} also suppresses the induction of IFN- α/β (31). For this, several mechanisms have been proposed. L^{pro} causes the degradation of NF κ B subunit p65/RelA (32), decreases the levels of interferon regulatory factor 3 (IRF3) and IRF7 (33), and it has been proposed to possess deubiquitinase (DUB) and deISGylase activities (34, 35). Having DUB activity is a common strategy for viruses to manipulate IFN signaling pathways (36). The 3C^{pro} of aphthoviruses has also been implicated in suppressing IFN- α/β production by cleaving NF κ B essential modulator (NEMO), an adaptor protein that is essential for activating NF κ B and IRF signaling pathways (37) and interfering with the JAK/STAT signaling pathways by manipulating the STAT1/STAT2 nuclear translocation (38).

Little is known about whether and how aphthoviruses suppress the cellular stress response. Here, we demonstrate that aphthoviruses can suppress the cellular stress response through the action of the viral protease L^{pro}, and we identify SG scaffold proteins G3BP1 and G3BP2 as new substrates of L^{pro}.

Results

The stress response pathway is suppressed during FMDV infection. To investigate whether FMDV antagonizes the stress response pathway, we infected LFPK cells that stably express the integrin $\alpha\beta$ 6 (LFPK $\alpha\beta$ 6 cells), the entry receptor of FMDV (39), with FMDV A12 and performed immune fluorescence staining to visualize SG formation (Fig. 1A). As a positive control, cells were treated with sodium arsenite, a known activator of the stress response pathway (Fig. 1B). SGs were visualized by anti-G3BP1 staining, while viral replication was monitored by staining for the capsid protein VP1. In LFPK $\alpha\beta$ 6 cells FMDV replication is relatively fast, virus production and release can be observed as early as 5 hrs post infection (hpi) (40). Consistently, we observed a clear accumulation of VP1 both at 4 and 6 hpi, indicative of efficient viral replication. Notwithstanding this, only a few G3BP1 foci were detected in the infected cells. In contrast, arsenite treatment of the cells led to extensive SG formation (Fig. 1B). These data show that FMDV infection does not induce SG formation. To investigate whether the virus actively suppresses SG formation, we infected LFPK $\alpha\beta$ 6 cells with FMDV A12 and added sodium arsenite during the last 30 min of infection (Fig. 1C). No SGs were observed in infected cells, whereas many SGs were observed in uninfected neighboring cells. Similar observations were made using IBRS-2 cells (Fig. 1D), providing further support that FMDV actively antagonizes SG formation.

FMDV and ERAV L^{pro} suppress SG formation when introduced into a recombinant EMCV containing an inactive stress antagonist. To determine whether the viral proteases are involved in suppressing SG formation, we generated recombinant EMCVs harboring FMDV L^{pro} or 3C^{pro} at the start of the ORF of EMCV-L^{Zn} (EMCV-FL and EMCV-F3C) (Fig. 2A). EMCV-L^{Zn} is a mutant EMCV (strain mengovirus) in which the antagonistic functions of the leader (L) protein are abolished by inactivating mutations in the zinc finger domain of L. This virus no longer suppresses the IFN- α/β or stress response (9, 18, 41, 42), and therefore provides a tool to study the antagonistic function of an heterologous viral protein (18, 43). The inserted proteases were flanked at their N-terminus by the first 6 amino acids of EMCV L to ensure efficient translation (44, 45), and an EMCV 3C^{pro} cleavage site at their C-terminus for efficient viral polyprotein processing. To determine whether the function of FMDV proteases is conserved in other aphthoviruses, we also constructed chimeric viruses encoding L^{pro} and 3C^{pro} of ERAV (EMCV-EL and EMCV-E3C). After recovering these chimeric EMCVs, we characterized viral replication kinetics by RT-qPCR analysis of EMCV viral RNA (vRNA) levels at various time points (Fig. 2B). As described previously (18, 41), mutation of the zinc-finger domain of EMCV L slightly reduced vRNA levels. Heterologous expression of either FMDV or ERAV L^{pro} (EMCV-FL and EMCV-EL) rescued viral replication to levels similar as those of wild-type EMCV, whereas expression of 3C^{pro} (EMCV-F3C and EMCV-E3C) did not affect vRNA levels.

Figure 1: FDMV suppresses SG formation. (A) LFPK $\alpha\text{v}\beta 6$ cells were infected with FMDV-A12, fixed at 4 and 6 hpi and immunofluorescence staining was performed for the viral capsid protein VP1 (green) and the SG marker G3BP1 (red). (B) LFPK $\alpha\text{v}\beta 6$ cells were treated with 500 μM sodium arsenite for 30 min to induce SG formation (as previously described (24, 26)) which were subsequently visualized by immunofluorescence staining for the SG marker G3BP1 (red). (C) LFPK $\alpha\text{v}\beta 6$ cells were infected with FMDV-A12, treated with 500 μM sodium arsenite for the last 30 min to induce SG formation, and fixed at 4 hpi. Immunofluorescence staining was performed for the viral capsid protein VP1 (green) and the SG marker G3BP1 (red). (D) Similar as (C) but using IBRS-2 cells.

Subsequently, we infected HeLa cells with these recombinant EMCVs and visualized SGs at 6 hpi using immune fluorescence microscopy. The SGs were visualized using anti-G3BP1, anti-G3BP2 and anti-TIA-1 staining. Consistent with previous results (9), EMCV infection suppressed SG formation, while EMCV-L^{zn} has lost this ability (Fig 2C, top two rows). Compared to EMCV-L^{zn}, infection with EMCV-FL or EMCV-EL resulted in noticeably fewer and smaller SGs. In contrast, we observed no effect of 3C^{pro} on SG formation (Fig. 2C). Similar observations were made at 8 hpi (data not shown). Quantification of SG size and number of

Figure 2: Aphthovirus L^{pro} suppresses SG formation. (A) Schematic representation of viral genome of chimeric EMCV viruses that were constructed for this study. The endogenous stress response antagonist (Leader) has been inactivated by point mutations in its Zn-finger domain (C19A/C22A), and subsequently the genes encoding L^{pro} and 3C^{pro} were introduced at the 5' end of the EMCV open reading frame. (B) HeLa R19 cells were infected at MOI 10 with the indicated chimeric EMCVs and subsequently virus growth kinetics were determined using RT-qPCR analysis with EMCV specific primers. (C) HeLa R19 cells were infected at MOI 10 with the indicated chimeric EMCVs and fixed at 6 hpi. Subsequent immunofluorescence staining was performed against the SG markers G3BP1 (green), TIA-1 (red) and G3BP2 (cyan). (D) Quantification of (C) in which the number of SGs and SG size was analyzed for at least 50 cells per condition. One-way ANOVA with Bonferroni post-hoc test was used to determine statistical significance (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

SGs showed a consistent decrease in both parameters upon the heterologous expression of L^{pro} (EMCV-FL and EMCV-EL), but not upon the expression of 3C^{pro} (EMCV-F3C and EMCV-E3C) (Fig 2D). Intriguingly, the viruses that have the ability to suppress SG formation (EMCV-FL and EMCV-EL) also replicated to levels similar as wt EMCV, while the viruses that failed to do so (EMCV-F3C and EMCV-E3C) replicated to levels similar as EMCV-L^{Zn}. This suggests that the ability of L^{pro} to suppress SG formation, likely combined with its ability to suppress the type I IFN response, is beneficial to the replication of EMCV-FL and EMCV-EL.

To determine if the inhibitory effect of L^{pro} on SG formation is dependent on L^{pro}'s catalytic activity, we generated an EMCV encoding a catalytically inactive FMDV L^{pro} (EMCV-FL C51A) and investigated the ability of this virus to suppress SG formation (Fig 3). HeLa cells were infected with EMCV-L^{Zn}, EMCV-FL or EMCV-FL C51A and SGs were visualized by immune fluorescence staining against G3BP1, eIF3 and G3BP2. Infection with EMCV-FL C51A resulted in SG formation (Fig 3A) and the SGs were of usual size (Fig. 3B). Collectively, these data show that L^{pro}, via its catalytic activity, suppresses SG formation.

Figure 3: Effect of L^{pro} on SG formation is dependent on the catalytic activity of L^{pro}. (A) HeLa R19 cells were infected at MOI 10 with chimeric EMCV expressing L^{pro} (EMCV-FL) or a catalytically inactive L^{pro} (EMCV-FL C51A) and fixed at 6 hpi. SG formation was visualized by immunofluorescence staining for the SG markers G3BP1 (green), eIF3 (red) and G3BP2 (cyan). (B) Quantification of (A) in which the SG size was analyzed for at least 50 cells per condition. One-way ANOVA with Bonferroni post-hoc test was used to determine statistical significance (* p<0.05).

L^{pro} prevents SG formation in FMDV-infected cells. To investigate whether L^{pro} also suppresses SG formation in aphthovirus-infected cells, we utilized a recombinant FMDV strain in which the leader-coding region was deleted (leaderless virus, LLV)(46). We infected LFPK $\alpha\beta6$ cells with wild-type FMDV or LLV and visualized SG formation at 4 hpi. Virus replication was monitored via staining for the capsid protein VP1, while SGs were visualized by anti-G3BP1

staining (Fig 4A). Similar amounts of VP1 were observed in cells infected with wild-type FMDV or LLV, indicating efficient viral replication irrespective of the presence or absence of L^{pro}, consistent with previous results (46). Infection with wild-type FMDV did not induce SG formation (Fig 4A and Fig 1). In contrast, infection with FMDV LLV triggered SG formation (Fig. 4A) and, upon quantification, we observed a consistent difference in the number of SG positive cells and the number of SGs per cell between wild-type FMDV-infected cells or LLV-infected cells (Fig 4B). These data demonstrate that FMDV LLV cannot block SG formation. Although we cannot formally exclude the involvement of some RNA element in the deleted region, our data suggests that L^{pro} is needed to suppress SG formation during FMDV infection.

Figure 4: Leaderless FMDV is unable to suppress SG formation. (A) LFPK $\alpha\beta6$ cells were infected with FMDV-A12 or leaderless FMDV-A12 (LLV), fixed at 4 hpi and immunofluorescence staining was performed for the viral capsid protein VP1 (green) and the SG marker G3BP1 (red). (B) Quantification of (A) in which the percentage of SG-positive infected cells and the number of SGs per cell were determined in at least 50 cells. One-tailed t-test was used to determine statistical significance (** p<0.01, *** p<0.001).

L^{pro} does not affect PKR signaling. We investigated whether aphthovirus proteases have an effect upstream of SG assembly, at the level of stress response signaling. To this end, we assessed the phosphorylation of PKR and eIF2 α , using flow cytometry. Infection of HeLa cells with EMCV-L^{Zn} is known to activate PKR (43), which results in the phosphorylation of eIF2 α and a subsequent halt in translation. Indeed, we observed increased levels of phosphorylated PKR and phosphorylated eIF2 α in EMCV-L^{Zn} infected cells (Fig 5A). The recombinant EMCVs expressing L^{pro} (EMCV-FL or EMCV-EL) or 3C^{pro} (EMCV-F3C or EMCV-E3C) also failed to suppress PKR or eIF2 α phosphorylation (Fig. 5B), indicating that neither L^{pro} nor 3C^{pro} affect PKR signaling, at least in HeLa cells infected with chimeric EMCV viruses.

Figure 5: L^{pro} does not inhibit PKR signaling. HeLa R19 cells were infected at MOI 10 with EMCV or EMCV-L^{Zn} (A) or the indicated chimeric EMCVs (B) and fixed at 6 hpi. Subsequent flowcytometry staining was performed for dsRNA and p-PKR or dsRNA and p-eIF2 α . Graphs represent the level of p-PKR or p-eIF2 α in dsRNA positive (infected) cells. Dashed line indicates level of p-PKR or p-eIF2 α in EMCV-L^{Zn} infected cells.

SG scaffold proteins are cleaved during aphthovirus infection. Many viruses target SG scaffold proteins to antagonize SG formation. Therefore, we investigated whether SG scaffold proteins are cleaved during FMDV infection. LFPK $\alpha\beta 6$ cells were infected with wild type FMDV-A12 or A12-LLV and the lysates were subjected to western blot analysis using G3BP1 antibody. Infection with wild-type FMDV, but not LLV, resulted in the cleavage of G3BP1 (Fig 6A). The predicted molecular weight of G3BP1 is 52 kDa, but we consistently observe the protein migrating at ~70 kDa. Upon FMDV infection, we detected two cleavage products that migrate at an apparent molecular weight of ~65 kDa and ~43 kDa (but which thus likely are smaller), but this was not observed in cells infected with FMDV LLV. A ~65 kDa cleavage product was first detected at 4 hpi and both cleavage products are clearly visible at 6 hpi. Collectively, our data suggest that G3BP1 is cleaved at multiple positions during FMDV infection. We also tested the lysates with antibodies against other human SG scaffold proteins, such as TIA-1 and eIF3, but unfortunately these antibodies did not show reactivity with swine proteins.

To investigate whether the ability to cleave SG scaffold proteins is conserved among aphthoviruses, we next studied the integrity of G3BP1, as well as G3BP2 and TIA-1, in ERAV-infected cells. To this end, HeLa cells were infected with ERAV or coxsackievirus B3 (CVB3, genus enterovirus), a control virus that is known to cleave G3BP1, but not G3BP2 or TIA-1 (24, 25, 47) (Fig 6B). Upon infection with ERAV, cleavages of G3BP1 and G3BP2, but not TIA-1, were observed (Fig. 6B). For G3BP1, we observed a large cleavage product that migrated at ~65 kDa and a doublet that ran at ~40 kDa. This ~40 kDa doublet was not observed in FMDV-infected cells. Meanwhile we barely detected a ~43 kDa cleavage product in ERAV-infected cells, which we did observe in FMDV-infected cells. Thus, FMDV infection (Fig. 6A) and ERAV infection (Fig. 6B) induced similar – but not identical – cleavages of G3BP1. G3BP2, like G3BP1, consistently migrated at ~70 kDa, but has a predicted molecular weight of only 54 kDa. Upon ERAV infection, we observed one G3BP2 cleavage product, running at ~65 kDa (although in reality this product may be smaller).

Next, we investigated whether aphthovirus proteases directly cleave G3BP1 and G3BP2, or indirectly by triggering apoptosis and activation of cellular caspases (Fig 6C). We infected HeLa cells with ERAV and treated them with pan-caspase inhibitor Q-VD-PH (Q-VD) to block caspase activity. While cleavage of PARP, a known caspase substrate, was inhibited by Q-VD (as demonstrated by the reduced amount of PARP cleavage product), ERAV-induced G3BP1 and G3BP2 cleavages were unaffected. Induction of apoptosis, by treatment with actinomycin D (Act. D), also induced cleavage of G3BP1 and G3BP2. Importantly, these caspase-mediated G3BP1 and G3BP2 cleavage products had a different molecular weight than those observed during ERAV infection and were inhibited by addition of Q-VD. Taken together, these results suggest that cleavage of G3BP1 and G3BP2 in aphthovirus-infected

cells is the result of a direct intervention of a viral protease, rather than activation of cellular caspases.

Figure 6: SG scaffold proteins are cleaved during aphthovirus infection. (A) LFPK $\alpha\beta 6$ cells were infected with FMDV-A12 or leaderless FMDV-A12 (LLV) and cells were lysed at indicated times p.i.. Western blot analysis was performed for G3BP1, GAPDH and the viral proteins VP1 and L^{pro}. (B) HeLa R19 cells were infected at MOI 10 with CVB3 or ERAV and cells were lysed at indicated times p.i.. Western blot analysis was performed for SG proteins G3BP1, G3BP2 and TIA-1. (C) HeLa R19 cells were infected with ERAV at MOI 10 or treated with 5 μ g/ml Actinomycin D. Subsequently cells were incubated for 16 hrs. in the presence or absence of 10 μ M Q-VD. Cell lysates were subjected to western blot analysis for G3BP1 and G3BP2, translation initiation factor 4G (eIF4G) and PARP. Arrows indicate full length proteins, black arrowheads indicate aphthovirus-induced cleavage products, grey arrowheads indicate cleavage products induced upon the induction of apoptosis (C).

L^{pro} cleaves G3BP1 and G3BP2. It has been suggested that EMCV infection triggers G3BP1 cleavage via 3C^{pro} (28). This would prevent us from investigating whether aphthovirus L^{pro} cleaves G3BP1 and G3BP2 by infecting cells with EMCV-FL and EMCV-EL. However, the cleavage of G3BP1 by EMCV is debated and could not be reproduced in another study (26). As such, we first infected HeLa cells with EMCV, EMCV-L^{zn} or CVB3 – as a positive control – and determined the integrity of G3BP1 and G3BP2 via western blot analysis (Fig. 7A). We did not detect cleavage of G3BP1 upon EMCV infection, using two different antibodies that recognize different epitopes within G3BP1. Our observations are consistent with reports that EMCV suppresses SG formation via its leader protein (9, 26).

To investigate which aphthovirus protease cleaves SG scaffold proteins, we infected HeLa cells with the EMCVs expressing L^{pro} and 3C^{pro} (EMCV-FL, EMCV-EL, EMCV-F3C and EMCV-E3C) and performed western blot analysis for G3BP1 and G3BP2. To compare the migration patterns of the cleavage products to those observed during aphthovirus infection, a lysate of ERAV-infected cells was run on the same gel (Fig. 7B). In agreement with the observation that leaderless FMDV failed to cleave G3BP1 (Fig. 6A), we observed that infection with EMCV expressing L^{pro} (EMCV-FL and EMCV-EL) resulted in the cleavage of G3BP1 and G3BP2 (Fig. 8A). A G3BP1 cleavage product migrating at ~65 kDa was observed during infection with EMCV-FL or EMCV-EL. A similar sized cleavage product prominently appeared during FMDV and ERAV infection. A ~43 kDa G3BP1 cleavage product, similar to the product that accumulated in cells infected with FMDV, also accumulated in cells infected with EMCV-FL. Such a ~43 kDa product was barely detected in ERAV-infected cells and did not appear in cells infected with EMCV-EL. Instead, a doublet cleavage product migrating at ~40 kDa was observed in ERAV-infected cells. We observed a similar doublet in cells infected with EMCV-F3C and EMCV-E3C, suggesting that 3C^{pro} can also cleave G3BP1. Intriguingly, such a ~40 kDa doublet was not observed in FMDV-infected cells. 3C^{pro} can also cleave G3BP1, albeit inefficiently and this had no effect on the integrity of SGs (Fig. 2). In ERAV-infected cells, we detected a G3BP2 cleavage product running at ~65 kDa (Fig. 6B and Fig. 7B). We observed a similar G3BP2 cleavage product, running at ~65 kDa, upon infections with EMCV-FL and EMCV-EL (Fig 7B), suggesting that L^{pro} cleaves G3BP2. Notably, infection with EMCV-FL resulted in a number of other bands that showed reactivity with the G3BP2 antibody, ranging from ~55 kDa to ~45 kDa. In particular, infection with EMCV-FL and EMCV-EL induced a product of ~45 kDa (indicated with *), that was not observed during ERAV infection. We did not observe cleavage of G3BP2 upon infection with either EMCV-F3C or EMCV-E3C. Together, these data suggest that L^{pro} has a major impact on the integrity of G3BP1 and G3BP2 in infected cells.

To gain more insight into the timing of G3BP1 and G3BP2 cleavage by L^{pro}, we next infected HeLa cells with EMCV-FL for different durations and performed western blot analysis of G3BP1 and G3BP2 (Fig 7C). The ~65 kDa G3BP1 cleavage product can be observed at 6 and 8 hpi. Meanwhile, we observed the ~43 kDa fragment at 8 hpi, suggesting that the first cleavage event allows the second cleavage event to occur. This sequential cleavage was also seen in FMDV-infected cells (Fig. 6A). A G3BP2 cleavage product, running at ~65 kDa, was first observed at 6 hpi but was more clearly visible at 8 hpi (Fig 7C). As anticipated, infection with an EMCV encoding the catalytically inactive FMDV L^{pro} (EMCV-FL C51A) failed to cleave G3BP1 and G3BP2 (Fig 7C). Collectively, our observations strongly suggest that L^{pro} is responsible for cleaving SG scaffold proteins in aphthovirus-infected cells.

To determine the location of the L^{pro} and 3C^{pro} cleavage sites in G3BP1, we co-expressed N-terminally Flag-tagged G3BP1 with EGFP-tagged L^{pro} or 3C^{pro} (Fig 7D). Under these conditions, we observed cleavage patterns of G3BP1 that resembled those observed in FMDV- and ERAV-infected cells and upon infection with the recombinant EMCVs. As the Flag-tag is located at the N-terminus of G3BP1, it can be concluded that the L^{pro} cleavage site yielding a ~65 kDa product is located in the C-terminal part of G3BP1. A second FMDV L^{pro} cleavage site, yielding a ~43 kDa fragment – and also the 3C^{pro} cleavage sites, yielding a ~40 kDa doublet – are located close to the middle of the protein, but slightly more towards the C-terminus. We also co-expressed Flag-tagged G3BP2 with EGFP-tagged L^{pro} (Fig 7E). We observed G3BP2 cleavages that were highly similar to those observed upon infection with ERAV, EMCV-FL and EMCV-EL. This suggests that the major L^{pro} cleavage site in G3BP2 is located in the C-terminal part of the protein. Notably, we did not observe the extra ~45 kDa cleavage product that was apparent upon infection with EMCV-FL or EMCV-EL, supporting the idea that this product is an artefact. We observed multiple cleavage products migrating around ~55 kDa upon overexpression of FMDV L^{pro}, similar as in cells infected with EMCV-FL. These products were not observed upon infection with EMCV-EL (Fig. 7B) and also not upon overexpression of ERAV L^{pro}. The approximate L^{pro} and 3C^{pro} cleavage sites in G3BP1 and G3BP2 – based on the apparent molecular weights of the cleavage products – are schematically represented in Fig. 7F. Collectively, these results indicate that the major cleavage products of G3BP1 and G3BP2, migrating at ~65 kDa, are generated upon cleavage of the C-terminus of these proteins. The additional G3BP1 and G3BP2 cleavage products – produced by FMDV L^{pro} and migrating at ~43 kDa and around ~55 kDa, respectively – are generated upon cleavage in the C-terminal half of the protein, close to the middle of the protein. The cleavage sequences of some substrates of L^{pro} are known, but we were not successful in identifying the cleavage sites in G3BP1 and G3BP2. Nevertheless, the cleavage sites of aphthovirus L^{pro} in G3BP1 clearly differ from that of enterovirus 3C^{pro} (Fig 6B).

Figure 7: Aphthovirus L^{pro} and 3C^{pro} cleave SG proteins. (A) HeLa R19 cells were infected at MOI 10 with EMCV, EMCV-L²ⁿ or CVB3 and cells were lysed at indicated times p.i.. Western blot analysis was performed with two different antibodies against G3BP1 (recognizing different epitopes) and G3BP2. (B) HeLa R19 cells were infected at MOI 10 with the indicated chimeric EMCVs or ERAV and cells were lysed at 8 hrs. (ECMV) or 10 hpi (ERAV). Subsequent western blot analysis was performed for G3BP1 and G3BP2. * indicates additional (artificial) G3BP2 cleavage product (C) HeLa R19 cells were infected at MOI 10 with EMCV-FL or EMCV-FL C51A and cells were lysed at the indicated timepoints. Subsequent western blot analysis was performed for G3BP1 and G3BP2. (D&E) Hek293T cells were co-transfected with a Flag-tagged G3BP1 or G3BP2 and a EGFP-tagged viral protease as indicated. 16 hrs. post transfection, cells were lysed and subjected to western blot analysis for FLAG and EGFP. Arrows indicate full length proteins, black arrowheads indicate L^{pro}-induced cleavage products, grey arrowheads indicate 3C^{pro}-induced cleavage products. (F) Schematic representation of approximate cleavage sites, based on apparent molecular weight of the cleavage products, in G3BP1 and G3BP2 for the different aphthovirus proteases. A region in G3BP2 containing multiple FMDV L^{pro} cleavage sites is indicated by ■.

Discussion

Over recent years, SGs are increasingly seen as important antiviral signaling platforms (2, 4–7). Several molecules involved in RLR signaling and stress response signaling have been found to localize in SGs (5, 8–10). Many viruses interfere with stress responses and the formation of SGs. Several picornaviruses belonging to the *Enterovirus* and *Cardiovirus* genus have been shown to actively suppress the stress response pathway (9, 24–26), but limited knowledge was available for members of the *Aphthovirus* genus. Here, we provide evidence that aphthoviruses suppress SG formation through L^{pro}. This papain-like protease is well known for its ability to cleave translation initiation factor eIF4G and thereby shuts off host translation (29). We showed that SG formation is suppressed in cells infected with wildtype FMDV but not in a leaderless mutant FMDV. Heterologous expression of L^{pro} of FMDV or ERAV in a mutant EMCV lacking its own leader protein, also suppressed SG formation. L^{pro} did not affect PKR activation and eIF2 α phosphorylation. Instead, western blot analysis showed that SG scaffold proteins G3BP1 and G3BP2 were cleaved in aphthovirus-infected cells. Similar cleavages were observed in cells infected with mutant EMCV expressing either L^{pro} and upon co-expression of Flag-tagged G3BP1/2 with L^{pro}. Collectively, our data demonstrate an essential role of L^{pro} in suppressing SG formation in aphthovirus-infected cells. The L^{pro}-mediated cleavage of SG scaffold proteins may underlie the inhibition of SG formation, although it cannot be excluded that a yet unidentified cleavage of other cellular protein(s) involved in stress signaling or SG formation also contributes to this inhibition. Besides dsRNA-dependent and PKR-mediated SG formation, also SG formation induced by sodium arsenite, which induces stress via a ROS-dependent mechanism via a different kinase (HRI), was inhibited. This lends support to the idea that L^{pro} has an inhibitory effect on formation and/or stability of SGs by cleaving essential SG components.

Cleavage of SG components likely also underlies the inhibition of SG formation by enteroviruses. Enterovirus infection results in formation of small SGs, which disappear as infection progresses. This observation has been attributed to cleavage of G3BP1 by enterovirus 3C^{pro} (24). However, recently evidence was presented that enterovirus 2A^{pro} rather than 3C^{pro} is responsible for suppression of SG formation in enterovirus-infected cells (48). Expression of 2A^{pro}, but not 3C^{pro}, of several enteroviruses was shown to inhibit SG formation induced by sodium arsenite or heat shock. Furthermore, infection of a recombinant EV-A71, in which 2A^{pro} activity was inactivated, led to the formation of typical SGs. The mechanism by which 2A^{pro} suppresses SG formation has not been elucidated, but these findings challenge the importance of cleavage of G3BP1 as an antagonizing mechanism to inhibit SG formation. Enterovirus 3C^{pro} does not cleave G3BP2 (25). Possibly the simultaneous cleavage of both G3BP1 and G3BP2 by L^{pro} is required to suppress SG formation. In line with this, it has been shown that SG formation is blocked in cells in

which G3BP1 and G3BP2 are depleted, but not in cells lacking only one of the G3BPs (49). Alternatively, the G3BP1 cleavage product generated by L^{pro}, which differs from that of enterovirus 3C^{pro}, may have a dominant negative effect on SG formation. The exact mechanisms used by aphthovirus L^{pro} and enterovirus 2A^{pro}, both of which are considered as “security proteins” (50), to suppress SG formation remains to be elucidated.

We found a strongly reduced amount of G3BP1 containing SGs in cells infected with wildtype FMDV. This observation is in agreement with previous observations that some small SGs that contain TIA-1 and Sam68, but lack G3BP1, are formed in FMDV-infected cells (51, 52). These small SGs resemble the small “atypical” SGs observed in cells expressing enterovirus 2A^{pro} (25, 48). These atypical SGs consist of cellular mRNAs of which translation is halted due to the cleavage of eIF4G by 2A^{pro} (25, 48). There is conflicting literature about the composition of these atypical SGs. Some studies have shown that these atypical SGs contain specific SG markers, i.e. TIA-1, TIAR and Sam68, but lack other SG markers, G3BPs and eIFs (48, 53), whereas others have shown that G3BP1-containing SGs are formed during enterovirus infection (24, 54) or upon overexpression of enterovirus 2A^{pro} (25). Upon infection with recombinant EMCV expressing either FMDV L^{pro} or ERAV L^{pro}, we observed small SGs containing TIA-1, G3BPs and eIF3. Although it is unclear what the exact composition is of atypical SGs, it seems plausible that enterovirus 2A^{pro} and aphthovirus L^{pro} can induce the formation of atypical SGs via their shared ability to inhibit host cell translation.

SG formation is induced upon the activation of eIF2 α kinases such as PKR and PERK. While enterovirus infection induces PKR activation, leading to an increase in both p-PKR and p-eIF2 α levels (55, 56), FMDV infection was shown to block activation of this pathway (57), possibly via 3C^{pro}-dependent lysosomal degradation of PKR (57). It has also been reported that the titers of FMDV LLV can be rescued in PKR knock-out cells (58), hinting to a possible role for L^{pro} in suppressing PKR signaling. We failed to observe any inhibitory effect on PKR signaling in cells infected with recombinant EMCV expressing either L^{pro} or 3C^{pro}. This may be a limit of our experimental setup to express these proteases outside the context of aphthovirus infection; e.g. it may suggest that antagonizing PKR signaling requires the action of multiple aphthovirus proteins. Nevertheless, we observed a clear inhibitory effect of L^{pro} on SG formation in cells infected with the chimeric EMCV-L^{pro}, independent of any effect on PKR signaling. Obviously, this does not rule out the possibility that effects of L^{pro} and/or 3C^{pro} on PKR signaling also contribute to the inhibition of SG formation in aphthovirus-infected cells, where this inhibition seems to be more pronounced than in cells infected with EMCV-L^{pro}. Multiple complementary SG inhibitory mechanisms may co-exist in aphthovirus-infected cells, one of which is mediated by FMDV L^{pro}.

Recently, G3BP1 was reported to bind to the IRES of FMDV and function as a translation inhibitor, and it was shown that FMDV 3C^{pro} induced the cleavage of G3BP1 (59). Interestingly both the N-terminal and C-terminal G3BP1 cleavage product maintained the negative effect on translation (59). In our study, we observed that FMDV and ERAV 3C^{pro} can cleave G3BP1 to some extent when expressed through recombinant EMCV. While this cleavage did not affect SG formation, it might thus affect viral translation.

In this study, we used a recombinant EMCV lacking its stress antagonist, the leader protein, to express heterologous proteinases of FMDV and ERAV. It has been suggested by others that EMCV suppresses SG formation through the 3C^{pro}-mediated cleavage of G3BP1 (28). As reported previously for both EMCV and TMEV (9, 26), we show that leader-inactivated EMCV (EMCV-L^{2m}) failed to suppress SG formation, whereas no SGs are observed in cells infected with wildtype EMCV. The leader of EMCV lacks proteolytic activity and no cleavage of either G3BP1 or G3BP2 was observed in EMCV infected cells. Thus, our data challenge the suggestion that EMCV 3C^{pro} suppresses SG formation by cleaving G3BP1.

In conclusion, in this study we present evidence that aphthoviruses actively suppress SG formation through the proteolytic activity of L^{pro}, and we identified two SG scaffold proteins, G3BP1 and G3BP2, as new targets of L^{pro}. These results add aphthoviruses to a growing list of viruses that prevent SG assembly to antagonize the stress response. These viruses include several flaviviruses (e.g. Hepatitis C virus, Dengue virus and West Nile virus), which use viral subgenomic RNA or nonstructural proteins to sequester SG proteins such as G3BP1, TIA-1 and TIAR to prevent SG formation (13, 14), as well as alphaviruses such as Semliki-forest virus (SFV), which prevents SG formation by sequestration of G3BP1 and G3BP2 by its nsP3 protein (15–17). Although these examples all hint to an important antiviral role of SGs, their exact role as a platform for antiviral signaling is yet poorly understood and more studies are warranted to potentially develop new tools to fight virus infection.

Materials and Methods

Cells and viruses. HeLa R19, BHK21 and Hek293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS (V/V). LFPK $\alpha\beta$ 6 cells (40) were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the PIADC. These cells were maintained in minimal essential medium (MEM) supplemented with 10% FCS (V/V) and 1% antibiotics and non-essential amino acids. BHK21 cells used for FMDV propagation were maintained in MEM supplemented with 10% FCS (V/V), 10% tryptose phosphate broth, 1% antibiotics and non-essential amino acids. FMDV A12-WT (wild type) was generated from the full-length serotype A12 infectious clone, pRMC35

(60) and A12-LLV2 (leaderless virus) was derived from the infectious clone lacking the Lb coding region, pRM-LLV2 (46). Viruses were propagated in BHK-21 and concentrated by polyethylene glycol precipitation, titrated on BHK-21 cells, and stored at -70°C. ERAV (NM-11/67 strain) (gift from D. Rowlands and T. Tuthill (University of Leeds, Leeds, United Kingdom) (61)) was obtained after passage on HeLa R19 cells and subsequently concentrated by ultracentrifugation through a 30% sucrose cushion at 140,000xg for 16 hours in a SW32Ti rotor and stored at -80°C. Recombinant EMCVs were generated by cloning the genes of interest into the XhoI/NotI restriction sites from the pM16.1-VFETQG-Zn infectious clone that was described previously (43), the Strep2 tag was omitted in the viruses used in this study. Viruses were recovered by transfection of run-off RNA transcripts into BHK-21 cells. Upon total CPE, viruses were concentrated by ultracentrifugation (as described for ERAV) and stored at -80°C.

Expression plasmids. The FMDV L^{pro} gene was obtained by PCR from the pM16.1 Mengo-FL infectious clone (45) using the following oligonucleotides 5'-CTCGAGCTGACACTGTACAACGGTGAG-3' and 5'-GCGGCCGCTTTGAGCTTGCGTTGAACCTTG-3', the FMDV 3C^{pro} gene was obtained by PCR using the following oligonucleotides 5'-AAAAAC TCGAGAGTGGTGCCCCACCG-3' and 5'-AAAAGCGGCCGCTCGTGGTGTGGTTCGGG-3' and the pBind-3C-VP16 FMDV plasmid (62) as template. The ERAV L^{pro} and 3C^{pro} gene were obtained by PCR from ERAV viral RNA (NM-11/67 strain) using the following oligonucleotides 5'-CTCGAGATGGACAAATTCTTGCAAAAAGAAAAAC-3' and 5'-GCGGCCGCTTCTCATACCTCTGATGTAAC-3' for L^{pro}, for 3C^{pro} the following oligonucleotides were used 5'-AAAAC TCGAGACTGGTGTGCCAGCAACTG-3' and 5'-AAAAGCGGCCGCTGTTTCTGAGGGAGAGTTCGC-3'. The oligonucleotides encode flanking XhoI and NotI restriction sites that were used to ligate the PCR products into the desired plasmids. The 3C^{pro} genes were ligated into the pcDNA-GFP vector described previously (11). The L^{pro} genes were ligated into the pIRES-EGFP-MCS plasmid. This is an pIRES2-EGFP based vector, in which the multiple cloning site has been relocated after the EGFP, allowing expression of an N-terminal EGFP fusion protein under expression of an EMCV IRES. The plasmids encoding Flag-G3BP1 and Flag-G3BP2 have been described elsewhere (11).

Antibodies. The following antibodies were used for IFA staining procedures: FMDV VP1 (mouse monoclonal Ab 6HC4), α dsRNA (J2, English&Scientific consulting), α G3BP1 (clone 23/G3BP, BD biosciences), α G3BP1 (ARP37713, Aviva systems biology), α G3BP2 (A302-040A, Bethyl laboratories), α eIF3 (N-20, SantaCruz) and α TIA-1 (C-20, SantaCruz). Alexa Fluor 488, Alexa Fluor 594 and Alexa Fluor 647 conjugated secondary antibodies (Molecular Probes, Invitrogen) were used for detection. For flowcytometry staining we used the α dsRNA (J2, English&Scientific consulting), α p-PKR (E120, Abcam), α p-eIF2 α (E90, Abcam) antibodies

and Alexa488 or Alexa647 conjugated (Molecular Probes, Invitrogen) secondary antibodies. For western blot we used the antibodies described above against G3BP1, G3BP2 and TIA-1 and the additional antibodies: α PARP (#11835238001; Roche Diagnostics), α Flag (M2, Sigma), α GFP (OSE00003G, Invitrogen), α tubulin (DM1A, Sigma). Respective IRdye680 or IRdye800 conjugated secondary antibodies (LiCOR) were used for detection.

Immune Fluorescence Analysis (IFA). LF-PK $\alpha\beta$ 6 cells were grown on 12 mm glass coverslips and infected with FMDV WT or LLV (MOI 10) for 1 h at 37°C. After adsorption, cells were rinsed with acidic buffer (150mM NaCl in 20mM morpholineethane-sulfonic acid (MES) pH 6.0), before adding MEM and proceeding with the incubation at 37°C for the indicated durations. The cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100® (Sigma) in PBS, blocked with blocking buffer (PBS, 2% bovine serum albumin [BSA], 5% normal goat serum, 10 mM glycine) and then incubated overnight at 4°C with primary antibodies against FMDV VP1 (mouse monoclonal Ab 6HC4) and G3BP2 (A302-040A-Bethyl Laboratories, Inc, Montgomery, TX). Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes, Invitrogen) conjugated secondary Abs were used for detection of green and red signal respectively. Nuclei were visualized by DAPI staining included in ProLong Gold Antifade mounting media (Invitrogen). Cells were examined in a fluorescence microscope and the images were taken with Nikon DS-Qi1 digital camera and NIS-Elements Advance Research v3.00 software (Nikon Instruments, Inc, Melville, NY). Alternatively, HeLa R19 cells were grown on 12 mm glass coverslips and the next day infected with the indicated viruses (MOI 10). At the indicated time points cells were washed in PBS before being fixed in 4% paraformaldehyde for 30 min. Residual paraformaldehyde was washed away using PBS+10 mM glycine. Cells were permeabilized in PBS+0.1% Triton-X100 and subsequently incubated in blocking buffer (PBS+0.1% Tween-20+3% BSA) for 2 hours. All subsequent steps are performed in blocking buffer. Samples are incubated with primary antibody for 1 hour and incubated with secondary antibodies and DAPI for 30 min. After antibody incubations, samples were washed three times with PBS+0.1% Tween-20 and once in milliQ before being mounted on microscopy slides using FluorSafe (Calbiochem). Cells were examined by confocal microscopy (Leica SPE-II) and Leica Application Suite Advanced Fluorescence software (LAS-AF).

Quantification of SGs. We analyzed the number of SGs and their surface area in ~100 cells per condition, the combined total of 10-20 images, using ImageJ software. For each image, background signal was removed by creating a blurred duplicate and subtracting it from the original image. Subsequently, remaining diffuse (cytoplasmic) SG marker signal was removed via weak blurring, adjustment of the contrast settings and applying a black-and-white threshold. In the resulting image, the number and average surface area of the SGs (shown in black on a white background) were quantified. The used macro is available upon

request. Subsequent statistical analyses were performed using GraphPad Prism software. Error bars represent standard deviations, p-values were calculated using one-way ANOVA analysis with Bonferroni post-hoc test (infections with recombinant EMCVs) or a one-tailed t-test (FMDV infections).

Flow cytometry analysis. HeLa R19 cells were seeded in 12-wells plates (200,000 cells/well) and the next day infected with the indicated viruses (MOI 10). Six hpi cells were trypsinized and resuspended in FACS buffer (PBS + 1% BSA). Cells were fixed for 30 minutes in 2% paraformaldehyde in FACS buffer and subsequently fixed in ice-cold methanol for 10 minutes. All subsequent steps are performed in FACS buffer. Cells were stained with primary antibodies for 1 hour. Subsequently cells were washed three times and incubated in secondary antibodies, in the dark, for 30 min. Cells were washed three times and kept in 1% paraformaldehyde until analysis on FACS Canto II (BD biosciences) using BD FACS Diva software. Data analysis was performed using FlowJo software.

Western Blot analysis. HeLa R19 cells were seeded in 10 cm dishes and the next day infected (MOI 10) with the indicated viruses. At the indicated time points cells were released using trypsin, washed once in PBS and lysed in 100 μ l lysisbuffer (100 mM Tris pH 8.0, 1 mM EDTA, 50 mM NaCl, 1% NP40, protease inhibitor mix (Roche). Post nuclear lysate was obtained by centrifugation at 15000xg at 4 °C for 15 min. The amount of total protein in the lysates was determined using BCA assay (ThermoFisher). 100 μ g protein from the cleared cell lysates were resolved using reducing sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.2 μ m nitrocellulose membranes by wet electrophoretic transfer. Membranes were incubated 1h in blocking buffer (PBS + 0.1% Tween 20 + 2% BSA) and successively incubated overnight with primary antibodies diluted in blocking buffer and then for 30 min with respective secondary antibodies diluted in blocking buffer. Between and after the incubations, the membranes were washed three times with PBS+0.1% Tween-20. Finally, membranes were washed once with PBS and scanned using an Odyssey Imager (Li-COR). For the analysis of protein of FMDV-infected cells, 40 ng of protein was resolved by SDS-PAGE, transferred by western blot and secondary antibodies conjugated with horseradish peroxidase (Pierce) were used for detection of proteins. Following incubation with appropriate primary and secondary antibodies, protein bands were visualized using SuperSignal® West Dura Extended Duration Substrate (ThermoScientific, Rockford, IL, USA) according to the manufacturer's directions.

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Chapter 3

Irreversible Inactivation of ISG15 by a Viral Leader Protease Enables Alternative Infection Detection Strategies

Kirby N. Swatek ¹, Martina Aumayr ^{2,*}, Jonathan N. Pruneda ^{1,*}, Linda J. Visser ^{3,*}, Stephen Berryman ⁴, Anja F. Kueck ¹, Paul P. Geurink ⁵, Huib Ovaa ⁵, Frank J. M. van Kuppeveld ³, Tobias J. Tuthill ⁴, Tim Skern ², David Komander ^{1,#}

¹ Protein and Nucleic Acid Chemistry Division, Medical Research Council Laboratory of Molecular Biology, CB2 0QH Cambridge, United Kingdom

² Department of Medical Biochemistry, Max F. Perutz Laboratories, Vienna Biocenter, Medical University of Vienna, A-1030 Vienna, Austria

³ Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

⁴ The Pirbright Institute, GU24 0NF Pirbright, Surrey, United Kingdom

⁵ Department of Chemical Immunology, Leiden University Medical Centre, 2333 ZC Leiden, The Netherlands

* These authors contributed equally.

Corresponding author.

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Abstract

In response to viral infection, cells mount a potent inflammatory response that relies on ISG15 and ubiquitin posttranslational modifications. Many viruses use deubiquitinases and deISGylases that reverse these modifications and antagonize host signaling processes. We here reveal that the leader protease, Lb^{pro}, from foot-and-mouth disease virus (FMDV) targets ISG15 and to a lesser extent, ubiquitin in an unprecedented manner. Unlike canonical deISGylases that hydrolyze the isopeptide linkage after the C-terminal GlyGly motif, Lb^{pro} cleaves the peptide bond preceding the GlyGly motif. Consequently, the GlyGly dipeptide remains attached to the substrate Lys, and cleaved ISG15 is rendered incompetent for re-conjugation. A crystal structure of Lb^{pro} bound to an engineered ISG15 suicide probe revealed the molecular basis for ISG15 proteolysis. Importantly, anti-GlyGly antibodies, developed for ubiquitin proteomics, are able to detect Lb^{pro} cleavage products during viral infection. This opens avenues for infection detection of FMDV based on an immutable, host-derived epitope.

Significance

An understanding of the mechanisms by which viruses evade host immunity is essential to the development of antiviral drugs and viral detection strategies. Ubiquitin and ubiquitin-like modifications are crucial in cellular innate immune and infection responses and are often suppressed by viral proteins. We here identify a previously unknown mechanism of viral evasion. A viral protease, Lb^{pro}, removes ubiquitin and the ubiquitin-like protein ISG15 incompletely from proteins. While this strategy efficiently and irreversibly shuts down these modification systems, it enables repurposing of tools and technologies developed for ubiquitin research in virus detection. Specifically, we show that foot-and-mouth disease virus infection can be detected using an anti-GlyGly antibody developed for ubiquitin mass spectrometry research.

Introduction

Picornaviruses are nonenveloped, positive-strand RNA viruses that comprise small genomes but represent significant threats to human and animal health (1). For example, foot-and-mouth disease virus (FMDV) is a global, highly infectious animal pathogen that causes annual economic losses in livestock farming estimated between \$6.5 and \$21 billion US dollars (2). Infected cattle develop fever and vesicular lesions in their mouths and hooves; consequently, FMDV outbreaks lead to culling of entire herds in FMDV-free countries to prevent the spread of the virus (3).

Among the 14 mature proteins encoded by FMDV are two peptidase activities required for self-processing and viral proliferation (4, 5). The leader protease Lb^{pro} is a papain-like cysteine protease that has several roles (4, 6). First, Lb^{pro} cleaves its own C terminus, releasing itself from the nascent polyprotein. Second, Lb^{pro} also cleaves the two isoforms of eukaryotic initiation factor 4G (eIF4G), the inactivation of which diverts ribosomes to preferentially translate viral RNA via their internal ribosome entry site (4, 6). Third, Lb^{pro} was reported to subvert innate immune signaling pathways by acting as a deubiquitinase (DUB)(7).

Ubiquitination is a key signaling mechanism used by cells to detect and respond to viral infection (8). Various ubiquitin signals play important roles during the activation of transcriptional programs, such as the NF-κB and IFN response (8, 9). With the initiation of IFN signaling, the products of roughly 300 IFN stimulated genes (ISGs) mount an antiviral response (10). Among the ISGs are the ubiquitin-like modifier ISG15 and its assembly machinery consisting of UBE1L, UBE2L6/UbcH8, and the HECT E3 ligase HERC5 (11). ISG15 comprises two ubiquitin fold domains in tandem [an N-terminal ubiquitin-like domain (NTD) and a C-terminal ubiquitin-like domain (CTD)], and like ubiquitin, it is attached to Lys residues on target proteins via its C-terminal Gly-Gly (Gly156–Gly157) motif. Cotranslational attachment of ISG15 to viral capsid proteins inhibits virus assembly (12), and additional intra- and extracellular roles of ISG15 are also emerging (13, 14). Together, ubiquitin and ubiquitin-like modifications regulate most antiviral signaling cascades.

Viruses are well-known to counter these defenses and manipulate the regulatory machineries or the modifications themselves (8, 9, 15). For instance, Crimean Congo hemorrhagic fever virus (CCHFV), severe acute respiratory syndrome coronavirus, and Middle East respiratory syndrome coronavirus all encode proteases with dual deISGylase and DUB activity (16).

We here show that FMDV Lb^{pro} targets ISG15 with strong preference over ubiquitin and NEDD8 and characterize this specificity biochemically and structurally. We uncover a previously undescribed mechanism by which viruses interfere with the ubiquitin and ubiquitin-like systems. Unlike canonical deISGylases, Lb^{pro} does not target the isopeptide bond formed during attachment but selectively cleaves a peptide bond in the C terminus of ISG15, which results in incomplete removal of the modifier. A crystal structure of Lb^{pro} covalently bound to a specifically designed ISG15 suicide probe reveals the molecular basis of Lb^{pro} specificity and substrate promiscuity. Importantly, cleavage by Lb^{pro} exposes a GlyGly epitope on substrates of the modifier, and we show that this epitope can be detected in cells during FMDV infection with relative ease. Such mechanism- and activity-based detection strategy opens avenues for distinguishing infected from vaccinated animals and may help limit the economic impact of FMDV.

Results

Lb^{pro} Substrate Specificity and Cleavage. We used ubiquitin and ubiquitin-like model substrates (17) to characterize Lb^{pro} DUB activity *in vitro* (Fig. S1A) and surprisingly found that while the enzyme lacked robust DUB activity, it targeted ISG15 with high activity and specificity (Fig. 1A). Ubiquitin cleavage required 1,000-fold higher enzyme concentration (Fig. S1B). Kinetic analysis revealed deISGylase activity comparable with previously studied viral (18, 19) and human enzymes (20) (Fig. 1B and Fig. S1C). However, a number of standard measurements for deISGylation activity were negative. ISG15-derived suicide probes that covalently modify ISG15-reactive Cys proteases (21, 22) were unable to modify Lb^{pro} (Fig. 1C and Fig. S1D). Hydrolysis of ISG15 7-amido-4-methylcoumarin (AMC) by deISGylases, such as the viral ovarian tumor (vOTU) domain of CCHFV, leads to fluorescence, yet Lb^{pro} did not generate a fluorescent signal in this assay (Fig. S1 E and F). In contrast, slightly extended precursor forms of full-length proISG15 (amino acids 1–165; featuring an additional eight residues at the C terminus) or proISG15CTD (amino acids 79–165) were rapidly hydrolyzed by Lb^{pro} (Fig. 1D). Strikingly, mass spectrometry (MS) analysis of Lb^{pro}-treated mature ISG15CTD (amino acids 79–157) revealed a truncated ISG15 molecule; Lb^{pro} had cleaved ISG15 between Arg155 and the C-terminal Gly156–Gly157 sequence (Fig. 1 E and F).

This surprising result was corroborated in additional experiments. We compared the cleavage rates of pro-, mature-, and substrate-bound forms of ISG15 by intact MS analysis, revealing similar activity of Lb^{pro} against pro-ISG15 and mature ISG15 and slightly higher activity toward ISG15 linked to 5-tetramethylrhodamine (TAMRA)-labeled Lys-Gly dipeptide via an isopeptide bond (Fig. S1G). This showed that Lb^{pro} can process all forms of ISG15 present in cells.

Figure 1: Lb^{pro} substrate specificity and cleavage. (A) Specificity analysis of Lb^{pro} against ubiquitin and ubiquitin-like (SUMO1, NEDD8, ISG15) TAMRA substrates (Fig. S1). (B) Michaelis–Menten kinetics of Lb^{pro} as measured by ISG15-TAMRA cleavage. Error bars represent SD from the mean. (C) Suicide ISG15 probe assays were performed with Lb^{pro} and WT ISG15CTD probe. (D) Comparison of full-length proISG15 (amino acids 1–165) and proISG15CTD (amino acids 79–165) cleavage by Lb^{pro}. (E) Electrospray ionization MS of untreated and Lb^{pro}-treated mature ISG15CTD (amino acids 79–157). The difference in mass corresponds to loss of the Gly-Gly peptide (114 Da). (F) Schematic of ISG15 cleavage by a canonical deISGylase (Left) and Lb^{pro} (Right). deISGylases cleave isopeptide bonds, while Lb^{pro} cleaves the peptide bond C-terminal to Arg155, releasing a GlyGly dipeptide. All assays were performed in triplicate. NTD, N-terminal ubiquitin-like domain.

The cleavage site of Lb^{pro} contrasts all known deISGylases, which cleave ISG15 from substrates, such that both proteins are recycled to the same state as they were before modification (18, 19). In hindsight, these findings explain the inability of Lb^{pro} to react with the ISG15 suicide probe (in which the reactive warhead would extend beyond the active site) or generate a signal in the ISG15-AMC assay. AMC only fluoresces in the unmodified form, whereas Lb^{pro} cleavage produces a nonfluorescent GlyGly-modified AMC reagent (Fig. S2A).

At higher enzyme concentrations, ubiquitin and NEDD8 were also cleaved at an identical site between Arg74 and the C-terminal GlyGly motif, whereas SUMO1 was not cleaved (Fig. S2 B–D). Hence, Lb^{pro} removes ISG15 (as well as ubiquitin and NEDD8) in a manner that leaves a GlyGly remnant attached to a substrate Lys residue and renders the ubiquitin/ubiquitin-like protein incompetent toward additional rounds of conjugation.

Structure of Lb^{pro} Bound to ISG15. To decipher the mechanism of this unprecedented activity, we designed an ISG15 suicide probe specific for Lb^{pro}. As mentioned, the WT ISG15 suicide probe modified several viral proteases but was too long to modify Lb^{pro} (Fig. S2E). Removing the GlyGly motif from ISG15^{CTD} and replacing Arg155 with a Gly-like C-terminal propargyl warhead (Fig. 2A) generated an ISG15^{CTD}-ΔC probe that efficiently modified Lb^{pro} (Fig. 2B and Fig. S2E). This enabled purification, crystallization, and structure determination of a covalent Lb^{pro}~ISG15^{CTD}-ΔC complex at 1.5-Å resolution (Fig. 2 C and D, Fig. S3, and Table S1). As anticipated, the ISG15^{CTD}-ΔC probe had covalently modified the catalytic Cys51 of Lb^{pro} (Fig. S3). Individually, the Lb^{pro} protease and ISG15 domains were similar to previously determined structures (18, 23) (Cα rmsds of 0.609 and 0.274 Å, respectively).

The structure revealed how proteolytic cleavage of ISG15 is achieved (Fig. 2E and Fig. S3). Key interactions are formed via the ISG15 C terminus and also, via a hydrophobic surface centered on ISG15 Trp123 (Fig. S3). The positively charged residues of the ISG15 C terminus, Arg153 and Arg155 (the latter mutated to Gly in the ISG15^{CTD}-ΔC probe), are cradled by an acidic groove that consists of Asp49, Glu96, and Glu147 on Lb^{pro} (Fig. S3G). Furthermore, ISG15 Leu154 occupies a hydrophobic pocket commonly found in papain-like enzymes. Mutation of either Leu154 or Arg155 to Ala in proISG15 strongly reduced cleavage by Lb^{pro} (Fig. S3H). However, these C-terminal mutations do not prevent ISG15 binding, since the ISG15^{CTD}-ΔC suicide probe that lacks Arg155 was able to modify Lb^{pro} (Fig. 2 B and C). The importance of the C terminus explains, at least in part, the observed weak cross-reactivity with ubiquitin and NEDD8, which feature identical or similar C-terminal sequences, respectively, and also, the inability of Lb^{pro} to target other modifiers, such as SUMO1, which feature more divergent C-terminal sequences (24) (Fig. S3F). The observed interactions closely mimic those seen in the previously determined structure of Lb^{pro} carrying out self-processing of its own C terminus (23) (Fig. 2E and Fig. S3F).

Figure 2: Structure of Lb^{pro} bound to ISG15. (A) Schematic of an engineered Lb^{pro}-specific ISG15 suicide probe. (B) The ISG15^{CTD}-ΔC suicide probe forms a covalent complex with Lb^{pro} (indicated by ~). (C) SDS-PAGE of the purified Lb^{pro}~ISG15^{CTD}-ΔC complex compared with unmodified Lb^{pro}. (D, Left) Crystal structure of Lb^{pro} covalently modified with the ISG15^{CTD}-ΔC probe. (D, Right) Close-up view of the ISG15^{CTD}-ΔC modified catalytic cysteine of Lb^{pro}. The catalytic triad (Cys51, His148, Asp163) of Lb^{pro} and C-terminal residues of ISG15 (amino acids 152–154; Arg155 was replaced by the Gly-like warhead) are highlighted. (E, Left) Structure of Lb^{pro} (Protein Data Bank ID code 1qol) during self-processing in trans. The Lb^{pro} substrate positioned for trans self-processing (yellow; Lb^{pro}-1) and the catalytic Lb^{pro} (cyan; Lb^{pro}-2) are shown. (E, Center) Lb^{pro} C-terminal residues of the substrate (Lb^{pro}-1) are shown (Arg198, Lys199, Leu200, Lys201), and residues of the catalytic triad are shown as in D for Lb^{pro}-2. (E, Right) The Lb^{pro}~ISG15^{CTD}-ΔC and Lb^{pro} self-processing structures were overlaid. Gel-based assays in B were performed in triplicate.

Analysis of ISG15 Specificity. Another interaction site between ISG15 and Lb^{pro} centers on the conserved Trp123 of ISG15 (Fig. 3A and Fig. S4A), the main hydrophobic ISG15 surface used in other deISGylases (Fig. S4B) (18, 19, 22, 25). Lb^{pro} utilizes a corresponding hydrophobic ISG15 binding surface formed by Leu92, Pro99 in the $\alpha 3/\alpha 4$ loop, and residues, including Leu102 on the $\alpha 4$ helix to shape the overall structure of the binding site (Fig. 3A and Fig. S3C); mutations in these residues decrease Lb^{pro} activity toward ISG15 (Fig. 3B and Fig. S4C). Importantly, this loop is not involved in interactions used during self-processing (Fig. 2E), and consequently and consistently, mutants in the ISG15 binding site are neither affected in their ability to self-process nor affected in their ability to cleave eIF4G (Fig. S5). Hence, substrate and ISG15 binding sites are spatially separated.

In ISG15, mutation of Trp123 to Ala leads to a significant reduction of cleavage by Lb^{pro} (Fig. 3C). Trp123 is also a major difference between ISG15 and ubiquitin, where the structurally equivalent residue is Arg42 (Fig. 3D). Mutation of ubiquitin Arg42 to Trp in the context of an Met1-linked ubiquitin chain significantly reduced cleavage of this chain type by the Met1 linkage-specific DUB OTULIN (26) (Fig. S4D) but enhanced ubiquitin chain cleavage by Lb^{pro} (Fig. 3E and Fig. S4E). This reveals one component of how Lb^{pro} distinguishes modifiers.

Substrate Cleavage in Cells. Given the high level of Lb^{pro} activity against ISG15 *in vitro* (Fig. 1 A and B), we were curious whether this activity and noncanonical cleavage were detectable in a biological context. Transfection of FLAG-tagged ISG15 and the ISG15 assembly machinery (UBE1L, UBE2L6, and HERC5) led to robust ISGylation in HeLa cells. Treatment of these cell lysates with Lb^{pro} collapsed the ISGylated proteins, while free ISG15 remained seemingly unchanged (Fig. 4A and Fig. S6C). Serendipitously, we observed that ISG15 cross-reacted with a polyclonal antiubiquitin antibody (Fig. S6 A and B). Treatment with Lb^{pro} specifically removed this cross-reaction, indicating that a fraction of the polyclonal antibody detects an epitope spanning the identical C terminus of both modifiers (Fig. S6 A and B). Antibody cross-reactivity provided us with a tool to monitor ISG15 cleavage by Lb^{pro} in cell extracts. Indeed, when transfection cell lysates were probed with the polyclonal anti-ubiquitin antibody, the ISG15 cross-reactive band was no longer detected after Lb^{pro} treatment, indicating that Lb^{pro} had hydrolyzed the ISG15 C terminus (Fig. 4A, red box). The antiubiquitin blot also showed that ubiquitin modifications in Lb^{pro}-treated samples were only partially processed, confirming ISG15 preference in complex samples (Fig. 4A). This was in stark contrast to the CCHFV vOTU domain, which cleaves ubiquitin and ISG15 similarly (18, 19) and collapsed both types of signals to the same extent (Fig. 4A).

To further visualize Lb^{pro} activity, we exploited a previously developed antibody that recognizes GlyGly-modified Lys residues (27, 28). This anti-GlyGly antibody has so far only been used to enrich ubiquitinated tryptic peptides in MS applications (27, 28). Rewardingly,

Figure 3: Structural analysis of ISG15 specificity. (A, Left) The Lb^{pro}–ISG15^{CTD}– Δ C complex is shown as in Fig. 2D. (A, Right) Close-up view of the hydrophobic contact between Lb^{pro} (Leu92, Pro99, Leu102) and ISG15 (Trp123) (Fig. S4). (B) ISG15-TAMRA cleavage assays with Lb^{pro} point mutants in the hydrophobic patch. (C) Lb^{pro} cleavage assays with WT and W123A proISG15^{CTD}. (D, Left) Model of ubiquitin (Protein Data Bank ID code 1ubq) bound to Lb^{pro}. Ubiquitin (red) was superimposed onto ISG15^{CTD}. (D, Right) Close-up view of the analogous interface as in A. (E) Met1 diubiquitin cleavage assays with or without R42W mutation in the distal ubiquitin. Arrows in the schematics show the proteolytic site of cleavage (Fig. S4E). Assays in B, C, and E were performed in triplicate.

probing these same reactions with the anti-GlyGly antibody produced a smear of strong signals across a wide range of molecular weights in the Lb^{pro}-treated sample (Fig. 4A). These signals correspond to the Lb^{pro}-produced GlyGly-modified proteome.

Revealing Lb^{pro} Activity During Viral Infection. To assess the impact of Lb^{pro} activity during viral infection, we first exploited a chimera viral infection model (29). A mengovirus (a strain of encephalomyocarditis, a picornavirus that is closely related to FMDV) system was engineered, in which the Leader protein was inactivated by mutations and functionally replaced by WT Lb^{pro} or catalytically inactive Lb^{pro} Cys51Ala as a leader protease. We monitored Lb^{pro} activity in a time course with anti-ISG15, anti-ubiquitin, and anti-GlyGly antibodies (Fig. 4B and Fig. S6D). With inactive Lb^{pro}, ISG15 modifications were visibly increased 6–8 h postinfection, whereas no visible changes to total ubiquitin and no signal in the anti-GlyGly Western blot were apparent. In contrast, mengovirus with active Lb^{pro} resulted in a decrease in ISG15 signals, a slight decrease in ubiquitin signals, and importantly, the appearance of GlyGly-modified proteins at 6 and especially, 8 h postinfection (Fig. 4B and Fig. S6D). This indicates that, indeed, production of active Lb^{pro} by the virus leads to incomplete hydrolysis of ISG15 and ubiquitin from substrates, which can be visualized using available ubiquitin proteomics antibodies.

Importantly, Lb^{pro} activity can also be visualized during FMDV infection [e.g. using baby hamster kidney (BHK) cells transfected with FLAG-tagged ISG15 and the ISG15 conjugation system]. Before infection, transfection leads to robust FLAG signals, whereas anti-GlyGly signals are absent. FMDV infection induces collapsing of FLAG signals coinciding with the appearance of GlyGly-modified proteins. Within 4 h of FMDV infection, the anti-GlyGly antibody labels hundreds of proteins across a broad molecular weight range (Fig. 4C). We are not aware of a similar host cell-derived viral-induced epitope that is detectable with such relative ease. Importantly, this epitope originates from host proteins and cannot acquire mutations due to viral evolution.

Discussion

Lb^{pro} is a prime example of a viral protein that contributes to successful viral replication through multifunctional roles. In addition to previously known crucial activities during replication (4, 6), we now show how it also hinders antiviral signaling through removal of ISG15 and to a lesser extent, ubiquitin from proteins (Fig. 5). Importantly, its mechanism of incomplete cleavage irreversibly damages the modifiers, which can no longer be attached to proteins. This mechanism is conceptually similar to the activity of RavZ, a *Legionella* effector that hydrolyzes the C terminus of Atg8 ubiquitin-like modifiers involved in

Figure 4: Validation of Lb^{pro} substrate cleavage in cells. (A) Lb^{pro} cleavage assays of ISGylated proteins. HeLa cells were transfected with FLAG-ISG15 and the ISG15 conjugation machinery (UBE1L, UBE2L6, HERC5) to produce ISG15ylated proteins. (A, Left) Removal of ISG15 from substrates by Lb^{pro} was monitored using an anti-FLAG antibody. (A, Center) A polyclonal antiubiquitin antibody monitors ubiquitin chain signals but can also be used to monitor cleavage of the ISG15 C terminus by Lb^{pro} (Fig. S6 A and B) (red box). (A, Right) The catalytic activity of Lb^{pro} was also monitored using an antibody that recognizes GlyGly-modified Lys residues. Loading controls are in Fig. S6C. (B) Infection assays using an FMDV Lb^{pro}-mengovirus chimera. The catalytic activity of Lb^{pro} was monitored as in A, with the difference being that ISG15 was detected using an anti-ISG15 antibody (Left). A catalytically inactive FMDV Lb^{pro}-mengovirus (C51A) was used as a control. Fig. S6D shows loading controls. (C) FMDV infection time course using BHK fibroblast cells transfected with FLAG-ISG15 and the ISG15 conjugation machinery. Lb^{pro} catalytic activity was monitored with anti-FLAG (Top) and anti-GlyGly (Bottom) antibodies. All assays were performed in triplicate.

autophagy (30). Moreover, on the substrate side, Lb^{pro} activity precludes remodification of Lys residues, and their small GlyGly modification(s) may not alter protein function significantly (Fig. 5). The slight cross-reactivity with ubiquitin is likely important, since ubiquitin modifications are much more abundant, and it is hence difficult to delineate the origin of the observed GlyGly signatures. Nonetheless, these multifaceted traits highlight the importance of Lb^{pro} as a potent virulence factor (31). It is possible that other viruses and pathogens may use this elegant anti-inflammatory strategy. While the leader proteases of other picornaviridae are highly divergent on the sequence level, the highly related aphthovirus equine rhinitis A virus may also encode an enzyme that generates GlyGly epitopes, which could be tested using GlyGly epitope detection in infected samples.

Figure 5: Model for Lb^{pro} action against ISG15 and ubiquitin. Lb^{pro} preferentially targets ISG15 over ubiquitin, which results from an optimized hydrophobic ISG15 binding site. The acidic groove coordinates the C terminus of ISG15 into the active site of Lb^{pro} and enables cleavage between Arg and GlyGly of the modifiers. This has two consequences: it renders ISG15 or ubiquitin incapable of (re-) conjugation and leaves substrates modified with a GlyGly remnant on their Lys residues. GlyGly-modified Lys remnants can be detected using available antibodies, enabling infection detection strategies. NTD, N-terminal ubiquitin-like domain.

The here identified virus-induced GlyGly remnants on substrate proteins may lead to improvements in the detection of foot-and-mouth disease (FMD). Vaccination is critical to the control FMD outbreaks; however, it is difficult to distinguish vaccinated from infected animals. Current strategies rely on ELISA-based methods to detect antibodies against nonstructural virus proteins in serum. Our findings suggest that GlyGly-modified proteins could also be used in ELISAs to detect antibodies against this epitope. Detection of antibodies against GlyGly modifications signifies enzymatic activity of Lb^{pro} that would only be observed after viral infection and hence, distinguish infected from vaccinated animals. This utility of detecting FMDV infection may ease the economic burden imposed by FMD, particularly in developing countries, by providing a previously unrecognized biomarker for its detection.

Methods

Cloning and Protein Purification. ISG15 and Met1 diubiquitin were cloned into an His-tagged expression vector (32). The Lb^{pro} vector (23) was expressed and purified according to (33). For the ISG15^{CTD}-ΔC probe, ISG15 (amino acids 79–154) was cloned in frame into the intein/chitin binding domain pTXB1 vector. ISG15-intein was expressed and purified according to (17, 34). SI Methods has expression and purifications procedures for His-tagged ISG15 and Met1 diubiquitin.

Biochemistry Assays. ISG15-AMC assays were performed as described previously (18). Ubiquitin/ubiquitin-like TAMRA assays were performed according to (35). ISG15-TAMRA reagent was used to determine Michaelis–Menten kinetics (additional details are in SI Methods). Cleavage assays of pro-ISG15^{CTD} and mature ISG15^{CTD} were performed under the same conditions as the TAMRA assays. ISG15 probe assays were also performed under these conditions. SI Methods has condition details. MS analysis was performed according to (36).

Crystallography. The Lb^{pro}~ISG15^{CTD}-ΔC complex was purified by anion chromatography (Resource Q) and dialyzed into 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, and 5% glycerol. After dialysis, the complex was concentrated to 4 mg/mL and set up at a 1:1 protein:precipitant ratio in a sitting drop vapor diffusion format. Crystals grew in 2.0 M ammonium sulfate, 0.1 M Na citrate, pH 5.6, and 0.2 M K/Na tartrate and were cryoprotected in mother liquor containing 25% glycerol. Crystallographic data were collected at the Diamond Light Source synchrotron (Table S1).

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Supplementary information

Figure S1: Analysis of Lb^{pro} substrate cleavage and efficiency. (A) Schematic of ubiquitin (Ub) and ubiquitin-like FP cleavage assays using TAMRA-labeled substrates. (B) Ubiquitin-TAMRA cleavage by Lb^{pro} at increased enzyme concentrations. (C) ISG15-TAMRA cleavage assays by viral proteases. Viral proteases include Lb^{pro}, Lb^{pro} C51A, and CCHFV vOTU. Positive (+) and negative (−) controls correspond to fully cleaved and uncleaved TAMRA reagents, respectively. (D) Suicide probe assays were performed with CCHFV vOTU at two concentrations of the WT ISG15^{CTD} probe. (E) ISG15-AMC cleavage assays with Lb^{pro}. Cleavage of ISG15-AMC was measured by fluorescence intensity (FI). Lb^{pro} cleaves ISG15-AMC, but the produced GlyGly-AMC remains nonfluorescent (Fig. S2A). (F) ISG15-AMC cleavage assays as in E with CCHFV vOTU. In contrast to Lb^{pro}, robust cleavage of ISG15-AMC by CCHFV vOTU was detected. (G) Comparison of proISG15^{CTD}, mature ISG15^{CTD}, and ISG15^{CTD}-TAMRA (isopeptide-linked ISG15) cleavage rates using intact MS. All assays were performed in triplicate. mFP, measured fluorescence polarization.

Figure S2: Characterization of Lb^{pro} ubiquitin and ubiquitin-like cleavage. (A) Analysis of ISG15-AMC by electrospray ionization MS without and with incubation of CCHFV vOTU and Lb^{pro}. The deconvoluted spectrum is shown, and a diagram depicts the theoretical and observed Δ masses of cleavage. *Contaminant of ISG15-AMC synthesis; this mass corresponds to ISG15 lacking the most C-terminal Gly residue. (B) Ubiquitin, (C) NEDD8, and (D) SUMO1 were analyzed by electrospray ionization intact MS before and after incubation with Lb^{pro}. The deconvoluted spectrum is shown. A diagram shows the ubiquitin and NEDD8 cleavage site. SUMO1 cleavage was not observed. (E) Reactivity of viral proteases with WT and ISG15^{CTD}-ΔC suicide probes. The viral proteases include CCHFV vOTU, Dugbe protease, human rhinovirus serotype 2 2A^{pro} (HRV2 2A^{pro}), coxsackievirus serotype B4 2A protease (CVB4 2A^{pro}), and FMDV Lb^{pro}. Reactions were incubated at 37 °C and subsequently resolved by Coomassie-stained SDS-PAGE. All assays were performed in triplicate. NTD, N-terminal ubiquitin-like domain.

Figure S3: Structural analysis of the Lb^{pro}-ISG15^{CTD}-ΔC structure. (A) Residues of the Lb^{pro}-ISG15^{CTD}-ΔC crystal structure are shown in stick representation for the complex in the asymmetric unit covered by the 1.5-Å 2|Fo|-|Fc| electron density map (gray indicates contoured at 1.2σ). (B) Close-up view of the Lb^{pro}-ISG15^{CTD}-ΔC covalent linkage; 2|Fo|-|Fc| electron density is shown for the C terminus of ISG15 (Leu152, Arg153, Leu154, propargyl warhead), and the catalytic triad of Lb^{pro} is shown contoured at 1.2σ. (C) Topology diagram of Lb^{pro}. The catalytic residues are indicated with red stars, and the ISG15 hydrophobic binding pocket is indicated with a yellow circle. (D) Comparison of Lb^{pro}-ISG15^{CTD}-ΔC with (E) CCHFV vOTU-ISG15 complex (PDB ID code 3phx). Close-up view of the covalently modified active site Cys residues of Lb^{pro}-ISG15^{CTD}-ΔC and vOTU-ISG15 (red arrows). (F) Sequence alignment of the C-terminal residues of Lb^{pro} self-cleavage to ubiquitin (Ub) and ubiquitin-like proteins (ISG15, NEDD8, SUMO1). A green arrow denotes the site of Lb^{pro} self-cleavage, and amino acid numbers are labeled according to Lb^{pro}. (G) Ionic interactions of Lb^{pro} with the C terminus of ISG15. Electrostatic potential of Lb^{pro} is shown in surface representation. Red represents negative electrostatic potential, while blue is positive. (H) proISG15^{CTD} cleavage assays as in Fig. 1D. Point mutants in the ISG15 C terminus (L154A and R155A) were incubated with Lb^{pro}, resolved by SDS-PAGE, and stained with Coomassie. All assays were performed in triplicate.

Figure S4: Characterization of Lb^{pro} ISG15 specificity. (A) Sequence alignment of ISG15 across multiple species. Trp123 is highlighted with a blue star and is conserved across vertebrates, except zebrafish. Another important area of sequence conservation is the C terminus. (B) LIGPLOT representation of Trp123 (Trp121 of mice) ISG15 interactions with Lb^{pro} and viral deISGylases, including CCHFV vOTU [PDB ID code 3phx] and Erv (ERVV) vOTU [PDB ID code 5jze]. Trp123 is shown in orange, and residues that form hydrophobic interactions with this residue are shown as red fans. (C) proISG15^{CTD} (amino acids 79–165) cleavage assays with mutations in the hydrophobic patch that make contact with Trp123 of ISG15. (D) OTULIN cleavage of Met1 diubiquitin substrates (compare with Fig. 3E). (E) Intact MS analysis of Met1 diubiquitin cleavage assays with R42W mutation in the distal ubiquitin (Ub). All assays were performed in triplicate.

Figure S5: Mutations in the hydrophobic ISG15 binding site of Lb^{pro} do not impact self-processing and elf4G cleavage. (A) Schematic of *in vitro* translation assays. The indicated RNA was translated in rabbit reticulocyte lysates (RRLs) containing [35S] methionine. On translation, Lb^{pro} undergoes self-processing, removing itself from the nascent polyprotein (VP4/VP2). Liberated Lb^{pro} then targets elf4G for cleavage. (B) WT Lb^{pro} and (C) Lb^{pro} mutant (L92A, P99A, and L102A) VP4/VP2 RNA were incubated in [35S] methionine containing RRLs. Time courses of Lb^{pro} self-processing and elf4G cleavage were detected by autoradiography and anti-elf4G antibody, respectively. NTD, N-terminal ubiquitin-like domain.

Figure S6. A ubiquitin polyclonal antibody allows for detection of Lb^{pro}-dependent ISG15 cleavage. Full-length ISG15 (1–157 amino acids) was incubated with Lb^{pro} in a standard cleavage assay. The indicated reactions and time points were analyzed by Western blotting using (A) anti-ISG15 antibody (H-150; Santa CruzInc.) and (B) anti-ubiquitin antibody (Millipore). Detection of ISG15 cleavage by Lb^{pro} can be visualized using the polyclonal anti-ubiquitin antibody, which cross-reacts with the conserved C terminus of ISG15. On cleavage of the C terminus by Lb^{pro}, the antibody no longer recognizes ISG15. (C) Ponceau S-stained blots from Fig. 4A. (D) Ponceau S-stained blots from Fig. 4B.

Lb ^{pro} -ISG15-ΔC	
Data collection	
Space group	C2
Cell dimensions	
a, b, c, Å	114.19, 40.35, 58.05
α, β, γ, °	90, 91.82, 90
Resolution, Å	58.02 – 1.50 (1.53 – 1.50)
R _{merge}	0.044 (0.489)
I/σI	13.4 (2.2)
CC _{1/2}	0.999 (0.866)
Completeness, %	99.2 (98.4)
Redundancy	4.2 (3.9)
Refinement	
Resolution, Å	58.02 – 1.50
Unique reflections	42,227
R _{work} /R _{free}	16.7/18.6
No. of atoms	
Protein	1,881
Ligand/ion	49
Water	213
B factors	
Protein	24.2
Ligand/ion	60.8
Water	36.3
rmsds	
Bond lengths, Å	0.020
Bond angles, °	1.43

Table S1: Data collection and refinement statistics. Values in parentheses are for highest-resolution shell.

SI Methods

Cloning and Site-Directed Mutagenesis.The expression vector pET11d/FMDV (23) was used as a template for all site-directed Lb^{pro} mutants (amino acids 29–195). Human ISG15 (Cys78Ser) and Met1 diubiquitin constructs were cloned into the pOPIN-BHis-tagged expression vector (32). A codon-optimized ISG15 was obtained from GeneArt Gene Synthesis (Invitrogen) and cloned in frame to the intein/chitin binding domain pTXB1 vector (NewEngland BioLabs) using conventional cloning methods. Site-directed mutagenesis was performed according to the QuikChange method. Plasmids for cell transfection assays (FLAG-ISG15, UBE1L, UBE2L6, and HERC5 of human origin) were cloned into the pcDNA3.1 mammalian expression vector.

Protein Expression and Purification. Lb^{pro} was expressed and purified according to (33). His-tagged ISG15 and Met1 diubiquitin were expressed in *Escherichia coli* Rosetta2 (DE3) pLacI (Novagen). Cultures were grown to OD600 (0.6–0.8) and induced with 0.2 mM Isopropyl β-D-1-thiogalactopyranoside for 16 h at 18 °C. Cell pellets were resuspended in 30 mL of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM β-mercaptoethanol) and frozen at –80 °C. Thawed pellets were supplemented with an EDTA-free Complete Protease Inhibitor tablet (Roche), 1 mg/mL lysozyme, and 0.1 mg/mL DNaseI and incubated on ice for 10 min. The cell suspension was sonicated, and cell debris was cleared by centrifugation at 50,000×g for 30 min. The cleared lysate was incubated with TALON Superflow resin (GE Healthcare) for 10 min and washed with 1 L of lysis buffer by gravity flow. Protein was eluted with lysis buffer supplemented with 250 mM imidazole. Purified proteins were dialyzed for 16 h into lysis buffer to remove excess imidazole. ISG15-intein protein was expressed identically to His-tagged proteins but purified with chitin resin (New England BioLabs) as described previously (17, 34).

AMC Fluorescence Measurements. ISG15-AMC cleavage assays were performed as described previously (18) with minor modifications. In each reaction, 10 μL of 2 μM ISG15-AMC (2×concentration) was mixed with 10 μL enzyme (2×concentration) in AMC assay buffer (50 mM Tris, pH 8.0, 10 mM DTT). The reactions were monitored using λ_{ex}=350 nm and λ_{em}=450 nm.

Fluorescence Polarization Assays. All ubiquitin/ubiquitin-like TAMRA assays were performed according to (35). Briefly, enzymes and substrates were diluted in dilution buffer (50 mM Tris, pH 8.0, 10 mM DTT). Substrate concentrations remained constant at 150 nM, while enzyme concentrations varied based on cleavage efficiency. Cleavage assays were performed in a black 384-wellplate (Corning), and fluorescence polarization (FP) measurements were made with a 550 nm excitation filter and a 590 nm emission filter using a Pherastar plate reader (BMG Labtech). All measurements were compared with substrate only (negative) and 25 nM KG-TAMRA peptide (positive) reference standards.

Kinetics. ISG15-TAMRA was used as a substrate for determination of Lb^{pro} Michaelis–Menten kinetics. The change in FP on Lb^{pro} hydrolysis was measured in a black 384-well plate using a PheraStar plate reader (BMG Labtech): λ_{ex}=550 nm and λ_{em}=590 nm. Reactions were incubated at 25 °C in a total volume of 20 μL in FP kinetics buffer (25 mM Tris, pH 8.5, 2 mM DTT, 0.1 mg/mL BSA). In each well, 10 μL ISG15-TAMRA (2×finalconcentration) was mixed with 10 μL of 40 nM enzyme (2×finalconcentration). The ISG15-TAMRA concentration was varied from 0.1 to 20 μM. All reactions were performed in triplicate and in parallel. The change in FP was measured in the linear range over a period of 0–7 min, with readings recorded every 5 s. The FP measurements were plotted over time, and initial velocities of

ISG15-TAMRA cleavage were determined. Michaelis–Menten parameters were calculated using Prism 6 GraphPad Software.

proISG15 Cleavage Assays. The proforms of ISG15 full-length (1–165) or the CTD (79–165) were used in all cleavage assays. The concentration of proISG15 was held constant at 10 μM and incubated at 37 °C in all cleavage assays. Reactions were quenched through the addition of lithium dodecyl sulfate (LDS) samplebuffer at the indicated time points. Samples were resolved on 10–20% Tricine gels (Invitrogen) and stained with Instant BlueSafeStain (Expedeon).

Intact MS. Cleavage assays were performed similarly to the proISG15 cleavage assays described above. In ubiquitin (1–76 amino acids), NEDD8 (1–76 amino acids), and SUMO1 (1–97 amino acids) cleavage assays, the concentration of Lb^{pro} was increased to 1 μM to promote cleavage of these non-preferred substrates. Unless stated otherwise, reactions were incubated at 37 °C for 1h and quenched with 50% acetonitrile and 0.1% formic acid. Substrates were diluted to 1 pmol/μL before intact MS analysis. Samples were injected at a flow rate of 5 μL/min and ionized using a heated electrospray ionization (HESI-II) source before being analyzed by an Orbitrap mass analyzer (QExactive). Ionization, data collection, and deconvolution of rawfiles were performed according to (36).

ISG15 Suicide Probe Assays. ISG15 probe assays were performed with 20 or 40 μM ISG15 probes with 10 μM Lb^{pro} or vOTU at 37 °C in probe assay buffer (100 mM Tris, pH 8.0, 10 mM DTT). The reactions were quenched in LDS sample buffer at the specified time points and resolved on a 4–12% Bis-Tris gel before being visualized with Instant Blue SafeStain (Expedeon).

Preparation of Lb^{pro}-ISG15 Complex for Crystallization. For crystallography, 45 μM Lb^{pro} was mixed with 175 μM CTD-ISG15-ΔCprobe in a total volume of 1 mL. The reaction was incubated for 4h at 37 °C. Immediately afterward, the reaction was placed on ice. The resulting complex was purified by strong anion chromatography (Resource Q) at pH 8.0 using a linear salt gradient (0–500 mM NaCl). The reacted complex was pooled and dialyzed into 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, and 5% glycerol. After dialysis, the complex was concentrated to 4 mg/mL using a Vivaspin 500 3K MWCO concentrator and used for crystallization screening. The purity of the complex was assessed by a Coomassie-stained SDS-PAGE gel and estimated to be >95% pure.

Crystallization. Initial hits were obtained from a commercial screen (Hampton Research) in a sitting drop vapor diffusion format. Lb^{pro}-ISG15-ΔC complex was set up at a 1:1 protein:precipitant ratio. Crystals grew in 2.0 M ammonium sulfate, 0.1 M Na citrate pH

5.6, and 0.2 M K/Na tartrate. Crystals were cryoprotected in mother liquor containing 25% glycerol.

Data Collection, Structural Determination, and Refinement. Data were collected at the Diamond Light Source beamline I-04 at a wave length of 0.92819 Å with a PILATUS 6M-F detector. Integration of images was performed using MOSFLM (37) and scaled using Aimless (38). The structure was solved by molecular replacement in Phaser (39) using the apo Lb^{pro} [Protein Data Bank (PDB) ID code 1qol (23)] and ISG15^{CTD} [PDB ID code 3phx (18)] structures. Model building and refinement were performed using COOT (40) and PHENIX (41). Table S1 shows statistics. Structural figures were created using PyMol (<https://pymol.org/2/>). The pro-origami server (42) was used to build the topology diagram.

In Vitro Self-Processing Assays. Lb^{pro} intramolecular cleavage was monitored by *in vitro* translation assays (15). To this end, the PCITE-1d plasmid containing the sequence for the Lbpro VP4/VP2 or Lb^{pro} mutants (L92A, P99A, L102A) was linearized with SalI followed by *in vitro* transcription (43). Template DNA was digested with DNase, and RNA was purified by phenolchloroform extraction. Then, rabbit reticulocyte lysates were programmed with *in vitro*-transcribed RNA of the precursor protein Lb^{pro} VP4/VP2 in the presence of [35S] methionine (43), and aliquots were removed at the specified times. The reaction was stopped in stop solution [61% (vol/vol) 2×Laemmli sample buffer, 0.6 mM methionine–cysteine mix, 37% (vol/vol) dH₂O]. Radiolabeled proteins were detected by analysis on a 17.5% SDS/PAGE gel followed by autoradiography. Gels were incubated twice with 20 mL enhancing solution containing 1 M sodium salicylate and 45% methanol for 15 min before being dried on Whatman paper using an SAVANT slab gel drier and exposed to a CL-X Posure film (Thermo Scientific) for 18h at –80 °C. Subsequently, the film was thawed and developed using an AGFA Curix660. Intermolecular cleavage of endogenous eIF4G was monitored by analyzing 10 μL of translation mix on a 6% SDS/PAGE gel and subsequent Western blotting with anti-eIF4GI antibody (a gift from Robert Rhoads, Louisiana State University HealthSciences Center, Shreveport, LA).

Cell Culture, Transfections, and Incubation with Lb^{pro}. HeLa cells obtained from ATCC were grown to 80% confluency before transfection with the indicated plasmids as previously described (12). Cells were harvested and lysed in lysis buffer (50 mM Tris, pH 8.0, 4 M urea, 50 mM NaCl, 10 mM chloroacetamide, 1 mM EDTA, 1 mM PMSF, 0.1% Nonidet P-40, 25 mM sucrose, 5% glycerol). Cells were briefly sonicated to shear DNA and further supplement lysis. Cell debris was cleared by centrifugation at 21,000×g for 10 min at 4 °C. Unreacted chloroacetamide was quenched with 10 mM DTT to prevent alkylation of the Lb^{pro} active site cysteine. Total protein was quantified using Bradford methods. Before incubation with Lb^{pro}, the concentration of urea was diluted to 1M; 100 μL of 5 mg/mL total

protein was incubated with 2 μ M Lb^{Pro} for 30 min at 37 °C. Reactions were quenched with LDS sample buffer at the indicated time points. Samples were resolved on a 4–12% Bis-Tris gel (Invitrogen) before being transferred to nitrocellulose membranes by Western blotting. Anti-FLAG (Sigma-Aldrich, M2), anti-ISG15 (Sigma-Aldrich, SAB1408653), anti-ubiquitin (Millipore, 07-375), and anti-GlyGly (Lucerna Inc., GX41) primary antibodies were used in Western blots. All Western blots, unless stated otherwise, were incubated for 16h at a 1:1,000 primary antibody dilution in phosphate buffered saline with Tween 20 (PBS-T)+5% BSA. The primary antibody dilution for anti-FLAG Western blots was 1:10,000. After primary antibody incubation, Western blots were incubated at a 1:5,000 secondary antibody dilution overnight in PBS-T+5% milk prior to being visualized by chemiluminescence. All antibody incubation steps were performed at 4 °C.

Recombinant Mengovirus Infection Assays. HeLa-R19 cells were cultured in DMEM (Lonza) supplemented with 10% (vol/vol) FCS. Recombinant viruses were derived from the pM16.1-VFETQG-Zn infectious clone as described previously (29). In short, genes of interest were inserted between the XhoI/NotI restriction sites of the pM16.1-VFETQG-Zn infectious clone. Viruses were recovered by transfection of run-off RNA transcripts into BHK-21 cells. On total cytopathic effect, cells were subjected to two freeze–thaw cycles, the cell debris was pelleted at 4,000 $\times$ g for 15 min, and the virus was concentrated by ultracentrifugation through a 30% sucrose cushion at 140,000 $\times$ g for 16h in an SW32Ti rotor. HeLa R19 cells were seeded in 10-cm dishes (2.0 $\times$ 10⁶ cells per dish). The next day, cells were infected (multiplicity of infection=10) with the indicated viruses. At 2, 4, 6, or 8 h postinfection, cells were released using trypsin, washed once in PBS, and lysed in 100 μ L lysis buffer [100 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl, 1% Nonidet P-40, protease inhibitor mix (Roche)]. Postnuclear lysate was obtained by centrifugation at 15,000 $\times$ g at 4 °C for 15 min. The amount of total protein in the lysates was determined using BCA assay (ThermoFisher).

FMDV Infection Assays. BHK-21 cells grown to 90% confluency in 24-well plates were transfected with FLAG-ISG15 and the ISG15 machinery (UBE1L, UBE2L6, and HERC5) and grown overnight. Transfected cells were then infected with FMDV01 Kaufbeuren diluted in virus growth medium (BHK cell culture medium containing 1% FBS). After 1h, the FMDV inoculum was removed, the cells were washed once with viral growth medium, and then, they were incubated in virus growth medium for the remainder of the infection. At 1h intervals postinfection (up to 5h), duplicate wells were washed once with PBS, and the cells were lysed in lysis buffer (50 mM Tris, pH 7.5, 0.5% IGEPAL, 120 mM NaCl). The lysates were clarified by centrifugation, and total protein was quantified using BCA assay (ThermoFisher). Equal quantities of the lysates (12 μ g) were loaded onto 10% acrylamide gels and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes and

blotted with either FLAG M2 (Sigma-Aldrich) or GlyGly (Lucerna Inc.) primary antibodies and mouse secondary antibody diluted in blocking buffer (5% milk and 0.1% Tween in PBS). Blots were visualized by chemiluminescence.

Chapter 4

Dissecting distinct proteolytic activities of FMDV L^{pro} implicates cleavage and degradation of RLR signaling proteins, not its deISGylase/DUB activity, in type I interferon suppression

4

Linda J. Visser¹, Chiara Aloise^{1,#}, Kirby N. Swatek^{2,5,#}, Gisselle N. Medina^{3,8,#}, Karin M. Olek^{4,#}, Huib H. Rabouw^{1,8}, Raoul J. de Groot¹, Martijn A. Langereis^{1,8}, Teresa de los Santos³, David Komander^{2,6,7}, Tim Skern⁴, Frank J.M. van Kuppeveld^{1*}

¹Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

²Protein and Nucleic Acid Chemistry Division, Medical Research Council Laboratory of Molecular Biology, CB2 0QH Cambridge, United Kingdom

³United States Department of Agriculture, Agricultural Research Service, Foreign Animal Disease Research Unit, Plum Island Animal Disease Center, Orient, NY 11957, USA

⁴Department of Medical Biochemistry, Max Perutz Labs, Vienna Biocenter, Medical University of Vienna, A-1030 Vienna, Austria

⁵Department of Molecular Machines and Signaling, Max Planck Institute of Biochemistry, Martinsried, Germany

⁶Ubiquitin Signaling Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, AU

⁷Department of Medical Biology, The University of Melbourne, Melbourne, AU

⁸Present addresses: GNM is at Kansas State University College of Veterinary Medicine, Manhattan, USA; HHR is at the Hubrecht Institute, Utrecht, The Netherlands; and MAL is at MSD Animal Health, Boxmeer, The Netherlands.

These authors contributed equally, * Corresponding author.

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Abstract

The type I interferon response is an important innate antiviral pathway. Recognition of viral RNA by RIG-I-like receptors (RLRs) activates a signaling cascade that leads to type I interferon (IFN- α/β) gene transcription. Multiple proteins in this signaling pathway (e.g. RIG-I, MDA5, MAVS, TBK1, IRF3) are regulated by (de)ubiquitination events. Most viruses have evolved mechanisms to counter this antiviral response. The leader protease (L^{pro}) of foot-and-mouth-disease virus (FMDV) has been recognized to reduce IFN- α/β gene transcription; however, the exact mechanism is unknown. The proteolytic activity of L^{pro} is vital for releasing itself from the viral polyprotein and for cleaving and degrading specific host cell proteins, such as eIF4G and NF- κ B. In addition, L^{pro} has been demonstrated to have deubiquitination/deISGylation activity. L^{pro}'s deubiquitination/deISGylation activity and the cleavage/degradation of signaling proteins have both been postulated to be important for reduced IFN- α/β gene transcription. Here, we demonstrate that TBK1, the kinase that phosphorylates and activates the transcription factor IRF3, is cleaved by L^{pro} in FMDV-infected cells as well as in cells infected with a recombinant EMCV expressing L^{pro}. *In vitro* cleavage experiments revealed that L^{pro} cleaves TBK1 at residues 692-694. We also observed cleavage of MAVS in HeLa cells infected with EMCV-L^{pro}, but only observed decreasing levels of MAVS in FMDV-infected porcine LFPK α V β 6 cells. We set out to dissect L^{pro}'s ability to cleave RLR signaling proteins from its deubiquitination/deISGylation activity, to determine their relative contributions to the reduction of IFN- α/β gene transcription. The introduction of specific mutations, of which several were based on the recently published structure of L^{pro} in complex with ISG15, allowed us to identify specific amino acid substitutions that separate the different proteolytic activities of L^{pro}. Characterization of the effects of these mutations revealed that L^{pro}'s ability to cleave RLR signaling proteins but not its deubiquitination/deISGylation activity correlates with the reduced IFN- β gene transcription.

Author's summary

Outbreaks of the picornavirus foot-and-mouth disease virus (FMDV) have significant consequences for animal health and product safety and place a major economic burden on the global livestock industry. Understanding how this notorious animal pathogen suppresses the antiviral type I interferon (IFN- α/β) response may help to develop countermeasures to control FMDV infections. FMDV suppresses the IFN- α/β response through the activity of its Leader protein (L^{pro}), a protease that can cleave host cell proteins. L^{pro} was also shown to have deubiquitinase and deISGylase activity, raising the possibility that L^{pro} suppresses IFN- α/β by removing ubiquitin and/or ISG15, two posttranslational modifications that can regulate the activation, interactions and localization of (signaling) proteins. Here, we show that TBK1 and MAVS, two signaling proteins that are important for activation of IFN- α/β gene transcription,

are cleaved by L^{pro}. By generating L^{pro} mutants lacking either of these two activities, we demonstrate that L^{pro}'s ability to cleave signaling proteins, but not its deubiquitination/deISGylase activity, correlates with suppression of IFN- β gene transcription.

Introduction

A virally infected cell activates a plethora of antiviral responses. One of the best-known antiviral responses is the induction of type I interferons (IFN- α/β). Replication of the viral genome generates double-stranded RNA (dsRNA) replication intermediates that can be recognized by cytoplasmic RIG-I like receptors (RLRs). For example, picornaviruses, small (~30 nm) non-enveloped viruses with a positive-sense RNA genome, synthesize replication intermediates that are predominantly recognized by MDA5 (1–4). Upon recognition of viral dsRNA, MDA5 interacts with MAVS, which subsequently activates TRAF3 and TBK1. TBK1 phosphorylates the transcription factors IRF3 and IRF7, resulting in their activation and dimerization. Simultaneously, TRAF3 interacts with the IKK complex to activate the transcription factor NF- κ B. Upon activation, IRF3, IRF7 and NF- κ B translocate to the nucleus, where they induce expression of IFN- α/β and other proinflammatory cytokines. Subsequent IFN- α/β signaling via the type I IFN receptor (IFNAR) and the JAK-STAT pathway induces the expression of hundreds of interferon stimulated genes (ISGs) (reviewed in (5, 6)).

IFN- α/β gene transcription is extensively regulated by post-translational modification of RLRs and their downstream signaling proteins, including phosphorylation and ubiquitination. Ubiquitin is a 8.5 kDa protein that can be covalently linked through an ϵ -amino peptide linkage to lysine residues in target proteins. Within the RLR signaling pathway RIG-I, MAVS, TBK1, TRAF3, TRAF6 and IKK γ are ubiquitinated and this affects their molecular interactions, localization, stability, or activity (reviewed in (7, 8)). Ubiquitination of RLR signaling proteins can both positively and negatively regulate the signaling pathway, which allows for rapid fine-tuning of the innate immune response against viral infection (reviewed in (7–9)). Consequently, many viruses encode enzymes with deubiquitinating (DUB) activity to manipulate the RLR signaling pathway and thereby suppress expression of IFN- α/β (reviewed in (9)).

In addition to ubiquitin, there are multiple ubiquitin-like modifiers, which can also be attached to target proteins. Of special interest is ISG15, an IFN-induced modifier of 17.5 kDa comprised of two ubiquitin-like domains in tandem. The exact antiviral properties of ISG15 are not yet fully understood (reviewed in (10, 11)). Early work on ISG15 depended on mouse models and showed that expression of ISG15 protected mice from viral infection (12–

15). However, important biological and structural differences between ISG15 of murine and human origin have since been reported (16–19). More recently, a picture is emerging that proteins are ISGylated co-translationally, explaining why predominantly viral proteins and ISGs are ISGylated upon infection in humans (20). ISGylation of RLR signaling proteins has been reported, but the effect of these modifications on the outcome of the signaling pathway is still unclear ((21–24), reviewed in (11)). In addition, ISG15 has been reported to act as a cytokine (25, 26).

IFN- α/β signals in autocrine and paracrine ways to induce a tissue-wide antiviral state, thereby limiting viral spread. To establish infection in their host, it is essential for viruses to suppress both the RLR signaling pathway and the downstream signaling of IFN- α/β . Affecting protein levels of important signaling molecules, either via cleaving them or inducing their degradation, is a strategy commonly used by viruses to suppress antiviral signaling (27–30). One such example is the picornavirus foot-and-mouth disease virus (FMDV). FMDV is a member of the genus *Aphthovirus*, which also contains bovine rhinitis A and B viruses, and equine rhinitis A virus (ERAV). The genetic information on the FMDV RNA genome is translated as one polyprotein that is autocatalytically processed into the mature proteins, two of which have been shown to possess proteolytic activity and also been implicated in suppressing IFN- α/β induction (reviewed in(31)). The 3C^{pro}, the protease that processes the majority of cleavage sites on the polyprotein, cleaves NF- κ B essential modulator (NEMO), an adaptor protein that is essential to activate the NF κ B and IRF signaling pathways (32).

The second protease on the polyprotein implicated in suppressing IFN- α/β induction is L^{pro}, a papain-like cysteine protease located at the N-terminus of the polyprotein (33). Once synthesized, L^{pro} immediately frees itself from the growing peptide chain by autocleavage at its own C-terminus. L^{pro} then efficiently cleaves the two isoforms of eIF (eukaryotic initiation factor) 4G to reduce protein synthesis from cellular mRNA (34) and suppresses the induction of IFN- α/β via several mechanisms. L^{pro} has been shown to induce the degradation of NF- κ B subunit p65/RelA (35, 36), and decrease the levels of IFN regulatory factor 3 (IRF3) and IRF7 (37). Further, L^{pro} can also interact with ADNP, a negative regulator of transcription (38). In addition to cleaving or degrading important signaling molecules, L^{pro} possesses deubiquitinase (DUB) activity which has been proposed to modulate RLR signaling (39). A subsequent study demonstrated that L^{pro} should be predominantly regarded as a deISGylase rather than a DUB as biochemical evidence showed that L^{pro} has a 1000-fold higher affinity for ISG15 than for ubiquitin (40). Structural studies and biochemical studies have shown separate substrate binding sites on L^{pro} for the viral polyprotein, the isoforms of eIF4G as well as for ubiquitin and ISG15 (40–42), suggesting that it may be possible to uncouple the activities of L^{pro} by the introduction of specific amino acid substitutions.

We therefore set out to uncouple the different activities of L^{pro} to discover whether L^{pro} suppresses RLR signaling through its deISGylase/DUB activity or through its ability to cleave and degrade multiple RLR signaling proteins. In this work, utilizing encephalomyocarditis virus (EMCV) expressing FMDV L^{pro} (EMCV-L^{pro}), we identify MAVS and TBK1 as new L^{pro} substrates and determined the cleavage site in TBK1. By introducing specifically designed mutations into L^{pro}, we further identified residues that are important for either the cleavage/degradation of RLR signaling proteins or for its deISGylase/DUB activity, thereby uncoupling the two catalytic activities of L^{pro}. We demonstrate that cleavage/degradation of RLR signaling proteins, but not the deISGylase/DUB activity of L^{pro}, correlates with suppressing IFN- α/β gene transcription.

Results

FMDV L^{pro} reduces IFN- β mRNA induction when introduced into a recombinant EMCV containing an inactive stress antagonist. To study the effects of L^{pro} on the induction of type I IFN in picornavirus-infected cells, we used two previously generated recombinant viruses; EMCV-L^{zn}, which contains inactivating mutations in the zinc-finger domain of the Leader (i.e. EMCV's RLR signaling antagonist) (1, 43, 44), and EMCV-L^{pro}, which was derived from EMCV-L^{zn} and additionally encodes FMDV L^{pro} at the N-terminus of its polyprotein (Fig 1A) (45). We also constructed a similar recombinant EMCV carrying a catalytically inactive L^{pro} (i.e. EMCV-L^{pro} C51A) (46). To determine whether EMCV-L^{pro} can suppress IFN- β induction, we infected HeLa cells with EMCV, EMCV-L^{zn}, EMCV-L^{pro} or EMCV-L^{pro} C51A and determined the IFN- β mRNA levels over time via RT-qPCR analysis (Fig 1B). Consistent with previous studies (37, 39, 47), wt L^{pro}, but not L^{pro} C51A, reduced the induction of IFN- β mRNA approximately 10-fold, indicating that the catalytic activity of L^{pro} is needed to suppress RLR signaling. In conclusion, the viruses that we generated (EMCV-L^{pro} and EMCV-L^{pro} C51A) accurately mimic the suppression of RLR signaling by L^{pro} as previously reported for FMDV-infected cells (47), providing us a model system to determine the mechanism via which L^{pro} suppresses type I IFN induction.

L^{pro} cleaves the RLR signaling proteins MAVS and TBK1. L^{pro} has been reported to degrade important signaling proteins such as the p65 subunit of NF- κ B, IRF3 and IRF7 (35, 37). To determine whether L^{pro} targets additional RLR signaling proteins, we subjected cell lysates of HeLa cells infected with EMCV-L^{zn}, EMCV-L^{pro} or EMCV-L^{pro} C51A to Western Blot analysis for the signaling proteins MAVS, TBK1 and IRF3, as well as the known L^{pro}-substrates eIF4G and G3BP1 (Fig 2A). EMCV capsid proteins and tubulin served as infection and loading controls, respectively. Infection with EMCV-L^{pro}, but not EMCV-L^{pro} C51A, resulted in the rapid cleavage of eIF4G (from 4 hpi onwards) and the cleavage of G3BP1

We next focused our attention to identifying the cleavage site in TBK1. To this end, we overexpressed N-terminally FLAG-tagged TBK1 together with GFP-tagged L^{pro} and performed Western Blot analysis. As seen in Fig 2B, GFP-L^{pro} was able to cleave Flag-TBK1. We observed an α FLAG-reactive cleavage product migrating at ~90-95 kDa, the same apparent molecular weight as the cleavage product we observed in EMCV-L^{pro} infected cells (Fig 2A), suggesting that L^{pro} cleaves TBK1 at its C-terminus. We also co-incubated recombinant His-TBK1 with increasing amounts of recombinantly expressed L^{pro} and L^{pro} C51A (Fig 2C). The *in vitro* incubation of His-TBK1 with wt L^{pro} also resulted in a ~90-95 kDa α His-reactive cleavage product, confirming that L^{pro} cleaves TBK1 at its C-terminus and does not rely on other cellular factors. Incubation of His-TBK1 with catalytically inactive L^{pro} did not result in the formation of a cleavage product, confirming that the cleavage is dependent on L^{pro}'s proteolytic activity. Subsequently, we showed that L^{pro} also cleaves TBK1 of murine origin (Fig 2D), which suggests that L^{pro} cleaves TBK1 in a conserved region. We identified residues ₆₉₂KLK₆₉₄ – which localize at the very C-terminus of TBK1 and are well conserved between human, murine and porcine TBK1 – as a possible cleavage site (Fig 2E). Indeed, mutation of these residues prevented the cleavage of TBK1 by L^{pro} (Fig 2D), confirming these residues are the cleavage site.

Upon identifying the cleavage site in TBK1, we next investigated whether the cleavage of TBK1 by L^{pro} inhibits its function in the RLR signaling pathway. To this end, we generated cells in which the endogenous TBK1 gene is replaced with a TBK1 truncation mutant representative of the ~90-95 kDa cleavage product. We first generated HeLa TBK1 k.o. cells using CRISPR/cas9 technology and characterized the remaining RLR signaling capacity of these cells (Fig S1A+B). We found that depletion of TBK1 is not sufficient to fully impair RLR signaling, probably because of functional redundancy with IKK ϵ (Fig S1B). TBK1 k.o. cells have a ~10-fold lower IFN- β induction upon infection with EMCV-L^{Zn}, transfection of vRNA or upon overexpression of MAVS. As expected, IFN- β induction resulting from transfection of IRF3, which acts downstream of TBK1, was not affected in the TBK1 k.o. cells. Subsequently, we expressed full length TBK1 or TBK1 Δ 35aa, which represents the N-terminal cleavage product, in TBK1 k.o. cells (Fig S1C) and determined whether expression of TBK1 and TBK1 Δ 35aa restored IFN- β mRNA expression upon transfection of poly (I:C). Expression of full length TBK1 in TBK1 k.o. cells fully restored IFN- β mRNA induction (Fig S1D) and TBK1 Δ 35aa was similarly efficient in this (Fig S1E), indicating that the L^{pro}-generated N-terminal cleavage product is signaling competent.

Figure 3: TBK1 is cleaved in aphthovirus infected cells. (A) LFPK α v β 6 cells were infected with FMDV-A12 or leaderless FMDV-A12 (LLV) at MOI 10 and lysed at the indicated times postinfection. Lysates were subjected to Western blot analysis for MAVS, TBK1, IRF3, eIF4G, G3BP1, viral protein VP1 and tubulin. **(B)** HeLa R19 cells were infected with EMCV or ERAV at MOI 10 and lysed at indicated times post infection. Cell lysates were subjected to Western Blot analysis for TBK1 and tubulin. **(C)** HeLa R19 cells were infected at MOI 10 with the indicated viruses and lysed at 8 hpi. Cell lysates were subjected to Western Blot analysis for TBK1 and actin. **(D)** HeLa R19 cells were infected with ERAV at MOI 10. Subsequently, the cells were incubated for 16h in the presence or absence of 10 μ M Q-VD, a pan-caspase inhibitor. Cell lysates were subjected to Western Blot analysis for TBK1, PARP and tubulin.

TBK1 is cleaved during aphthovirus infection. To investigate whether TBK1, MAVS and IRF3 are cleaved during FMDV infection, we infected porcine LFPK α vb6 cells with wt FMDV-A12 or FMDV-A12 lacking L^{pro} (leaderless virus, A12-LLV). Western Blot analysis revealed cleavage of TBK1, but not of MAVS or IRF3, during wt FMDV-A12 infection (Fig 3A). TBK1 cleavage was observed from 4 hpi onwards upon infection with wt FMDV, but not upon infection with leaderless FMDV. The cleavage product had an apparent molecular weight of ~90-95 kDa, consistent with our previous observations of the size of this cleavage product. Although a MAVS cleavage product was not detected during FMDV infection, densitometry analysis revealed a strong and progressive decrease in the relative ratio of MAVS/tubulin from 2-6 hpi with wt FMDV compared to mock-infected cells, whereas only a small decrease was detected in leaderless-infected cells (MAVS/tubulin ratio is indicated in Fig 3A). This suggests that expression of L^{pro} induces degradation of MAVS, also in FMDV-infected cells. Consistent with our observations in EMCV-L^{pro} infected cells, we did not observe a decrease in IRF3 signal in FMDV-infected cells.

To investigate whether the cleavage of TBK1 is conserved amongst aphthoviruses, we infected cells with ERAV, the closest relative of FMDV. Infection with ERAV, but not EMCV, resulted in the cleavage of TBK1 (Fig 3B). However, the cleavage product was less prominent than for EMCV-L^{pro}, suggesting that cleavage was inefficient or infection was delayed. Notably, in our HeLa cells, ERAV displayed a replicative cycle of ~16 h. This is considerably slower than FMDV, which replicates in 6-8 hours. To study TBK1 cleavage by the two different L^{pro}s irrespective of variation in viral replication kinetics, we infected cells with EMCV-L^{pro} or an EMCV expressing ERAV L^{pro} (EMCV-ERAV L^{pro}), for which we previously determined the replication kinetics to be similar (45). Both viral proteases cleaved TBK1 resulting in a ~90-95 kDa cleavage product (Fig 3C). FMDV L^{pro} cleaved TBK1 more efficiently than ERAV L^{pro}, consistent with the results observed during infection with FMDV or ERAV (Fig 3A and 3B). Notwithstanding the differences between ERAV L^{pro} and FMDV L^{pro}, our data demonstrate that the ability to cleave TBK1 is conserved amongst these two aphthoviruses.

We also investigated the effect of the pan-caspase inhibitor Q-VD-PH (Q-VD) on TBK1 cleavage in ERAV-infected cells (Fig 3D). While addition of Q-VD decreased the cleavage of known caspase substrate PARP, the cleavage of TBK1 was unaffected. Collectively, these results demonstrate that L^{pro} directly cleaves TBK1 and that this activity is conserved amongst aphthoviruses.

A

B

Mutation	Affected domain	Phenotype	References
C ⁵¹ A	Catalytic site	Inactivation of catalytic activity (<i>in vitro</i> assays and ectopic overexpression)	46
I ⁸³ A/L ⁸⁶ A	SAP domain	Reduced DUB activity (ectopic overexpression) Failure to suppress IFN induction (<i>in infected cells</i>)	36, 37, 39
L ⁹² A	ISG15 binding domain	Reduced affinity for ISG15 and ubiquitin (<i>in vitro</i> assays)	40
L ¹⁰² A	ISG15 binding domain	Reduced affinity for ISG15 and ubiquitin (<i>in vitro</i> assays)	40
C ¹³³ S	eIF4G binding domain	Reduced affinity for eIF4G (<i>in vitro</i> assays)	48
L ¹⁴³ A	Substrate binding cleft	Broader specificity in binding pocket (<i>in vitro</i> assays)	49

Figure 4: Visualization and summary of the L^{pro} mutants used in this study. (A) Standard cartoon view of L^{pro} (grey) bound to ISG15 (blue) (PDB: 6FFA). L^{pro} L92 and L102 interact with ISG15 W123, this positions ISG15's C-terminus (R153, L154 and G155) in the substrate binding cleft of L^{pro}. *In this structure a C-terminal propargyl warhead replaced G155 (40). Residues which upon mutation were reported to affect L^{pro}'s structure or function are shown as colored sticks. Green: C51A inactivates L^{pro}'s catalytic activity; red: I83A or L86A reduce the DUB activity and IFN induction; pink: L92A and L102A reduce affinity for ISG15; orange: C133S reduces affinity for eIF4G; aquamarine: L143A predicted to open substrate binding pocket. Drawings were generated using PyMOL. (B) Overview of the reported effects of the introduced mutations in L^{pro} on the various proteolytic activities of L^{pro}, this includes references and the category of the underlying experimental evidence.

Construction of L^{pro} mutants to uncouple cleavage/degradation of RLR signaling proteins from its deISGylase/DUB activity. L^{pro} also possesses deISGylase and - to a lesser extent - DUB activity (39, 40, 42), and this latter activity was previously suggested to be important for L^{pro}'s ability to suppress RLR signaling (39). To investigate how L^{pro} reduces the induction of IFN- β gene transcription, we set out to uncouple these two abilities of L^{pro}. To this end, we introduced previously described mutations in the chimeric EMCV-L^{pro} and determined whether these mutations affect the deISGylase/DUB activity of L^{pro} and/or its ability to cleave/degrade RLR signaling proteins. A mutation in the SAP domain (I83A/L86A), was previously reported to abolish the ability of L^{pro} to suppress type I IFN expression, to degrade signaling proteins (i.e. NF- κ B p65, IRF3 and IRF7), and to disrupt its DUB activity (36, 37, 39), and was therefore included in our screening. Analysis of the crystal structure of L^{pro} bound to ISG15 suggested that L^{pro} residues L92, P99 and L102 are important for ISG15 binding (40). Fig 4A shows the structure of L^{pro} (grey) in complex with ISG15 (blue) and indicates the residues of ISG15 (W123 and R153, L154, G155) that interact with L^{pro}. Mutation of L92, P99 or L102 in L^{pro} reduced its affinity for ISG15 and impaired its deISGylase activity, without affecting eIF4G cleavage (40). Homology modeling showed that ISG15 and ubiquitin interact with the same surfaces of L^{pro} (40), suggesting that L^{pro}'s deISGylase activity also reflects its DUB activity. Two of these mutations (L92A and L102A) were introduced in EMCV-L^{pro}. In addition, we introduced the mutations L143A and C133S. Mutation C133S was reported to reduce the affinity of L^{pro} for eIF4G (48) whereas mutation of L143 to alanine with its shorter side-chain rescued polyprotein processing in the context of the additional mutation L200F (49). Based on the structure of L^{pro}, mutation L143A has been predicted to open up the catalytic pocket. Figure 4 shows the locations of the residues that are mutated in this study (Fig 4A) and summarizes the reported effects of these mutations on L^{pro}'s various proteolytic activities (Fig 4B).

Effect of L^{pro} mutations on cleavage and/or degradation of RLR signaling proteins. First, we determined the effect of the introduced mutations on L^{pro}'s ability to cleave or degrade RLR signaling proteins. We infected Hela cells with EMCV, EMCV-L^{pro} or the different EMCV-L^{pro} carrying the described mutations, lysed the cells at the indicated timepoints and subjected the lysates to Western Blot analysis for MAVS, TBK1, NF- κ B subunit p65, IRF3, eIF4G and G3BP1, as well as L^{pro} and EMCV capsid proteins (Fig 5A). Our data show that infection with the different L^{pro} mutant viruses resulted in L^{pro} expression and accumulation of EMCV capsid proteins from 6 hpi onwards, which is indicative of efficient infection. Interestingly, the introduced mutations in L^{pro} had different effects on the cleavage or degradation of RLR signaling proteins. Upon infection with EMCV-L^{pro} C133S, we observed a ~2 hr delay in eIF4G cleavage, consistent with a previous report (48). This mutation did not affect the cleavage/degradation of the various RLR signaling proteins. Mutation of L92 or L102 has been reported to reduce the activity of L^{pro} towards ISG15, without affecting eIF4G cleavage

(40). Indeed, infection with EMCV-L^{pro} L92A, resulted in cleavage of eIF4G, as well as all other L^{pro} substrates (i.e. MAVS, TBK1, NF- κ B p65 and G3BP1) (Fig 5A). Infection with both EMCV-L^{pro} L92A and EMCV-L^{pro} L102A resulted in efficient cleavage of RLR signaling proteins, confirming that these two L^{pro} mutants have the same proteolytic profiles (Fig 5B). Of note, the cleavage of eIF4G appears to occur faster by L^{pro} L92A and L102A compared to wt L^{pro}, although a more in-depth analysis is necessary to confirm a true difference in eIF4G cleavage kinetics. Importantly, both mutations allow us to separate the deISGylase/DUB activity of L^{pro} from its ability to cleave RLR signaling proteins. Serendipitously, we observed that L^{pro} carrying mutation L143A was strongly impaired in degrading NF- κ B p65 and cleaving MAVS and TBK1, while cleavage of G3BP1 and eIF4G was delayed but could be observed clearly at later timepoints (eIF4G cleavage is ~2 hr delayed, comparable to the delay observed for L^{pro} C133S). Mutation of L^{pro}'s SAP domain (I83A/L86A), which was previously shown to abolish degradation of NF- κ B p65 and DUB activity as well as to impair L^{pro}'s ability to reduce IFN- β mRNA expression (36, 39), also affected L^{pro}'s ability to cleave MAVS and TBK1. Overall, our data demonstrate that the mutations have differential effects on the cleavage/degradation of RLR signaling proteins. Importantly, we demonstrate that L^{pro} residues L92 and L102, which are essential for its deISGylase/DUB activity, are not essential for its ability to cleave/degrade RLR signaling proteins, indicating that the two different catalytic activities of L^{pro} can be uncoupled.

Effect of L^{pro} mutations on DUB and deISGylase activities. It has been previously reported that mutations L92A, L102A and I83A/L86A affect L^{pro}'s deISGylase/DUB activity (39, 40). To determine the effect of mutations L143A or C133S on these activities, mutant L^{pro}'s were expressed and purified from *E. coli* and *in vitro* catalytic activities towards ubiquitin-TAMRA and ISG15-TAMRA were measured (Fig 6A). L^{pro} C133S and L143A displayed wt-like activity towards ISG15 and ubiquitin. We next determined DUB activity of wt L^{pro}, L^{pro} C51A, C133S and L143A in cells. To this end, we transfected HEK-293T cells with a combination of HA-ubiquitin, FLAG-RIG-I and increasing amounts of GFP-L^{pro} encoding plasmids (0.1, 0.3 and 0.5 μ g), and visualized HA-tagged ubiquitinated proteins by Western Blot analysis (Fig 6B). FLAG-RIG-I, which was included to monitor the effects of L^{pro}-induced translational shut-off on the overexpressed proteins, was clearly detectable even at the highest level of L^{pro} (i.e. transfection of 0.5 μ g of L^{pro} plasmid), indicating that L^{pro}-induced translational shut-off did not significantly reduce the protein expression from the transfected plasmids. Both wt L^{pro} as well as the C133S and L143A mutants displayed DUB activity upon overexpression in cells, as indicated by the reduction of HA-tagged ubiquitinated proteins upon increasing L^{pro} levels. Notably, we observed ubiquitinated proteins upon transfection of low amount of L^{pro} C133S plasmid (0.1 μ g), although our *in vitro* data (Fig 6A) indicated that mutation C133S does not reduce the activity for ubiquitin. As the *in vitro* data are much more quantitative in nature, we consider the relatively decreased DUB activity of L^{pro} C133S

Figure 5: Mutation of L143 or SAP domain strongly reduces cleavage and/or degradation of RLR signaling proteins. (A) HeLa R19 cells were infected at MOI 10 with the indicated viruses and lysed at 2, 4, 6, 8 h p.i. Cell lysates were subjected to Western Blot analysis for MAVS, TBK1, NF-κB p65, IRF3, eIF4G, G3BP1, L^{pro}, EMCV capsid proteins and tubulin. **(B)** HeLa R19 cells were infected at MOI 10 with the indicated viruses and lysed at 2, 4, 6, 8 h p.i. Cell lysates were subjected to Western Blot analysis for MAVS, TBK1, NF-κB p65, IRF3, eIF4G, G3BP1, L^{pro}, EMCV capsid proteins and tubulin.

in comparison to L^{pro} wt or mutant L143A as observed in Fig 6B the result of variations in expression of the transfected plasmids.

Figure 6: Mutation of L143 or C133S does not affect DUB or delSCglase activities. (A) Ubiquitin-TAMRA or ISG15-TAMRA (substrates) were incubated with 10 μ M or 100 nM L^{pro} (wt or indicated mutant), respectively. The change in fluorescent polarization (mFP) was determined over 60 min, with readings recorded every 60 sec. The average trace of assays performed in technical triplicate is shown. Substrate only and KG-TAMRA peptide served as respective negative and positive controls. (B) HEK293T cells were transfected with 0.5 μ g pcDNA-FLAG-RIG-I, 0.5 μ g pcDNA-HA-ubiquitin and 0.1 - 0.5 μ g pIRES-GFP-L^{pro}. 16 h post transfection, cells were lysed and lysates subjected to Western Blot analysis for FLAG, HA, GFP and tubulin.

Thus far, our data showed that mutations C133S and L143A did not affect L^{pro}'s DUB activity towards a mono-ubiquitin fused to a fluorescent TAMRA molecule. To exclude the possibility that mutations C133S and L143A affect L^{pro}'s DUB activity towards other substrates, we determined its ability to cleave differently-linked di-ubiquitin molecules. Co-incubation of L^{pro} with di-ubiquitin molecules of different linkages indicated that L^{pro} preferentially targets K6- and K48-linked ubiquitin chains, displayed some activity towards K11-, K33-, K63-, and M1-linked chains, but at this enzyme concentration and incubation times has no activity towards K27- or K29-linked ubiquitin chains (Fig 7). Neither mutation L143A nor C133S

affected the ability of L^{pro} to cleave the di-ubiquitin molecules. Overall, our analysis shows that L^{pro} carrying mutation L143A or C133S has wt-like deISGylase and DUB activity. Importantly, as mutation L143A impairs L^{pro}'s ability to cleave/degrade RLR signaling proteins (Fig 5), but does not affect its deISGylase/DUB activity, introduction of this mutation also allows us to make a distinction between the two proteolytic activities of L^{pro}.

Figure 7: Mutation of L143 or C133 does not affect activity towards differently linked ubiquitin. Wild-type L^{Pro} and the L^{Pro} mutants (C133S, L143A) were incubated with each of the eight di-ubiquitin chain types for the indicated times, separated by SDS-PAGE, and visualized by silver-staining. The concentration of L^{Pro} used in the cleavage assays is indicated.

L^{pro}'s ability to reduce IFN- β mRNA levels correlates with its ability to cleave/degrade RLR signaling proteins, not with its deISGylase/DUB activity. Having characterized how the different mutations in L^{pro} affect its deISGylase/DUB activity or the cleavage/degradation of RLR proteins, we next set out to determine which mutants reduce the induction of IFN- β mRNA. We infected HeLa cells with EMCV, EMCV-L^{zn} or the different EMCV-L^{pro} mutants and determined the IFN- β mRNA and EMCV vRNA levels over time via RT-qPCR analysis (Fig 8). Wildtype L^{pro} as well as L^{pro} C133S, L92A and L102A consistently reduced the induction of IFN- β mRNA, while L^{pro} C51A and L143A were unable to do so (Fig 8A). The SAP domain mutant (EMCV-L^{pro} I83A/L86A) also failed to suppress IFN- β mRNA levels (Fig 8B), which is in agreement with observations in FMDV-infected cells (36, 39). Notably, infection with EMCV L^{pro} L143A, which displayed wt deISGylase/DUB activity but is strongly impaired in its ability to cleave/degrade RLR signaling proteins MAVS, TBK1 and NF κ B p65, failed to suppress the induction of IFN- β mRNA. In contrast, L^{pro} L92A and L102A, which are strongly impaired in their deISGylase/DUB activity (40) but not in their ability to cleave/degrade RLR signaling proteins, reduced IFN- β mRNA levels. These combined observations (summarized in Fig 9) demonstrate that cleavage/degradation of RLR signaling proteins, but not the deISGylase/DUB activity of L^{pro}, correlate with suppressing IFN- α/β gene transcription. Notably, L^{pro} C133S reduced the induction of IFN- β mRNA despite a 2h delay in eIF4G cleavage, indicating that the rapid eIF4G cleavage and the subsequent translational shut-off is not sufficient to suppress RLR signaling.

Discussion

FMDV suppresses IFN- α/β both at the mRNA and at the protein level (47), but the molecular mechanism underlying the reduced induction of IFN- α/β gene transcription (RLR signaling) is poorly understood. Both the DUB activity of L^{pro} as well as its ability to cleave/degrade RLR signaling proteins have been implicated in the suppression of RLR signaling (35, 37, 39). In this study, we identified MAVS and TBK1 as novel L^{pro} substrates and mapped the cleavage site in TBK1. Moreover, by introducing specific mutations we were able to separate L^{pro}'s deISGylase/DUB activity from its ability to target RLR signaling proteins. Using L^{pro} carrying either of these uncoupling mutations, we demonstrated that the cleavage/degradation of RLR signaling proteins, not the deISGylase/DUB activity, correlates with the ability to reduce IFN- β gene transcription. Collectively, our data strongly suggest that the ability of L^{pro} to cleave/degrade RLR signaling proteins is needed to reduce the IFN- β mRNA levels.

Figure 8: Reduction in IFN- β gene transcription correlates with cleavage and/or degradation of RLR signaling proteins. (A+B) HeLa R19 cells were infected at MOI 10 with the indicated viruses and cells were lysed at 8 hpi. Total RNA was isolated and used for RT-qPCR analysis for IFN- β and actin mRNA (upper panels), and EMCV vRNA (lower panels). The IFN- β levels are depicted as a fold induction compared to levels in mock-infected cells, after correction for actin mRNA levels. The EMCV vRNA is depicted as a copy number per cell, calculated from a plasmid standard. Error bars represent the SD. One-way ANOVA with the Dunnett post hoc test was used to determine statistical significance compared to the results for EMCV-L^{zn}-infected cells (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

We identified TBK1 as a new L^{pro} substrate and identified the cleavage site. We observed cleavage of TBK1 both in HeLa cells infected with EMCV-L^{pro} and in FMDV-infected LFPK cells, and we demonstrated that the L^{pro} cleavage site is located towards the C terminus of TBK1, more specifically in the coiled-coil 2 (CC2) domain. Previous work indicated that TBK1 Δ CC2 was signaling competent upon overexpression of TBK1. However, it was also shown that mutation of residues L693 and K694, which are located in the CC2 domain, abolishes IFN- β mRNA induction upon polyI:C transfection and VSV infection (50). L^{pro} cleaves TBK1 at these residues. Yet, we observed that the N-terminal cleavage product restored RLR signaling in TBK1 k.o. cells

upon poly(I:C) stimulation. Whether this implies that the L^{pro}-mediated cleavage of TBK1 does not contribute to the viral strategy to suppress RLR signaling and IFN-β gene transcription remains unknown. Unfortunately, it is very difficult to investigate the effect of TBK1 cleavage by L^{pro} on IFN-β gene transcription in infected cells, as L^{pro} also cleaves other RLR signaling proteins, i.e. MAVS (this study) and LGP2 ((51), see below), and the transcription factor NF-κB. Dissection of the effect of TBK1 cleavage on IFN-β gene transcription from the effect of MAVS, LGP2 and NF-κB cleavage would require the identification of all L^{pro} cleavage sites in these known targets followed by the generation of cells with cleavage-resistant versions of these proteins. It remains a question whether such an approach will yield conclusive answers as other RLR signaling proteins may be targeted by L^{pro}, which have not yet been identified. Hence, the relative importance of the L^{pro}-mediated cleavage of TBK1 for the viral suppression of IFN-β gene transcription remains unknown.

Apart from its role in RLR signaling, TBK1 has been suggested to be involved in autophagosome maturation. TBK1 was identified as a factor in the autophagosomal clearance of herpes simplex virus 1 and mycobacteria (52, 53). A recent report showed that TBK1 phosphorylates lipidated LC3 to prevent premature removal of LC3 from autophagosomal membranes by ATG4, thereby facilitating a unidirectional flow from the autophagosome to the lysosome (54). Many picornaviruses hijack autophagic pathways to generate sites for viral RNA replication and to facilitate non-lytic release of virions (55–61) Possibly, cleavage of TBK1 by L^{pro} facilitates the use of autophagy to aid viral infection and propagation.

L^{pro} also impacts MAVS integrity. A distinct MAVS cleavage product was observed upon infection with EMCV-L^{pro}. Remarkably, no MAVS cleavage product was observed in FMDV-infected cells, although MAVS levels progressively declined over time. The reason for this difference is unknown, but may be related to differences in human and porcine cells. Nevertheless, our data indicate that the integrity of MAVS is affected by L^{pro} in both cell types, suggesting this RLR signaling protein is targeted by FMDV to affect IFN induction. Of note, MAVS is known to localize to the peroxisomes and mitochondrion associated membranes of the ER (62, 63), in addition to its default localization on mitochondria. It remains to be established whether L^{pro} targets all forms of MAVS or specifically targets MAVS at one of these locations. TBK1 and MAVS are not the only RLR signaling proteins that are targeted by L^{pro}. It was previously reported that overexpression of L^{pro} induces the degradation of IRF3 and IRF7 (37), and that the p65 subunit of NF-κB is degraded in FMDV-infected cells (35). We also observed degradation of NF-κB p65 in EMCV-L^{pro} infected cells but we did not observe degradation of IRF3. Notably, degradation of IRF3 was also not observed in FMDV-infected cells. Whether IRF3 degradation is restricted to certain cell types or conditions, or merely is an artefact due to overexpression remains unknown.

It is remarkable that L^{pro}, comprising just 173 amino acids, can carry out several specific proteolytic activities on both the viral and cellular substrates as summarized in Figure 4 and 9. Previous work documented areas of the protease that are required for polyprotein processing (41, 49). These residues included L143 which is part of the P2 pocket that can interact with leucine residues in the substrate at the P2 position. In this work, L143 was identified as being involved in TBK1 and MAVS cleavage; however, its replacement by alanine affected neither the activity on eIF4G nor the deISGylase or DUB activities. Surprisingly, mutation of two residues of the SAP domain, (I83 and L86) also affected TBK1 and MAVS cleavage, even though they are separated by 20 Å (measured between the respective C_α atoms) with helix α4 lying between them. Nevertheless, it cannot be excluded that the SAP mutations cause some destabilization of L^{pro}, thereby explaining this mutant's defect in proteolytic activities. Further structural work will be required to understand how L^{pro} interacts with TBK1 and MAVS.

While this work was in progress, it was reported that LGP2, a factor that is essential for MDA5 activation, is cleaved by L^{pro} (51). The mutations in L^{pro} that impaired the reduction of IFN-β mRNA levels (L143A and mutations in the SAP domain) displayed an overall defect in the cleavage and/or degradation of each of the RLR signaling proteins we studied (i.e. MAVS, TBK1 and NF-κB p65). We anticipate that the cleavage of LGP2 is also likely impaired by introduction of these mutations. Our data suggest that expression of L^{pro} results in cleavage and/or degradation of multiple RLR signaling proteins (MAVS, TBK1, NF-κB p65, and most likely LGP2). The relative contribution of each cleavage event to the reduction in IFN-β gene transcription remains unknown. A search for other substrates of L^{pro} is of importance to further our understanding of the role and mechanism of how L^{pro} reduces IFN-α/β induction. Possibly, such a search may identify RLR signaling proteins that are cleaved earlier than the ones identified so far and may thereby have an influence on the early induction of type I IFN in infected cells.

The L^{pro} mutants that are defective in either the deISGylase/DUB activity or the cleavage/degradation of RLR signaling proteins allowed us to study which ability is needed to suppress RLR signaling. Mutation L143A, which rendered L^{pro} unable to reduce IFN-β mRNA levels, impaired the cleavage of RLR signaling molecules, but had no effect on the deISGylase or DUB activity of L^{pro}. Meanwhile, mutations L92A and L102A resulted in the opposite phenotypic effect; RLR signaling proteins were cleaved with similar efficiency as wt L^{pro}, but the deISGylase activity was significantly reduced by these mutations (40). Yet, these L^{pro} mutants still reduced IFN-β mRNA levels. Collectively, these data indicate that the activity of L^{pro} to cleave/degrade RLR signaling proteins, not its deISGylase/DUB activity, is important for reduction of IFN-β induction. In support of this hypothesis, Medina et. al. have just reported that impairment of the deISGylation activity of L^{pro} causes viral attenuation *in vitro* and *in vivo*, without affecting IFN or ISG mRNA expression levels (64).

It was previously suggested that the DUB activity of L^{pro} is important for the suppression of RLR signaling (39), which contrasts our findings. Importantly, most experiments by Wang et. al. relied on overexpression of L^{pro}, ubiquitin, and several targets proteins (i.e. RIG-I, TBK1, TRAF3 and TRAF6). A recent study showed that L^{pro} should be predominantly regarded as a deISGylase rather than a DUB, as biochemical evidence showed that L^{pro} has a 1000-fold higher activity towards ISG15 than ubiquitin (40). Given the weak DUB activity of L^{pro} *in vitro*, it remains to be established whether L^{pro} genuinely acts as a DUB in FMDV-infected cells under physiological conditions (i.e. without overexpression of components of the ubiquitination system or known ubiquitination target proteins). Previously, we found no differences in the levels of ubiquitinated proteins in cells infected with EMCV expressing wt L^{pro} or L^{pro} C51A, whereas the levels of ISGylated protein were decreased in cells infected with EMCV-L^{pro} (40), suggesting that L^{pro} predominantly acts as a deISGylase in infected cells.

It is well established that certain viruses (i.a. adenoviruses, herpesviruses and nidoviruses) rely on viral proteases with DUB and deISGylase activity to suppress the induction of IFN-α/β (9). FMDV L^{pro} is a papain-like protease and thus L^{pro} is best compared to other virally encoded papain-like cysteine proteases that suppress IFN-α/β gene transcription. Members of the order Nidovirales (i.e. coronaviruses and arteriviruses) encode one or two papain-like cysteine protease (PLP), referred to as PL^{pro}, or PLP1 and PLP2 when the virus encodes two PLPs. In addition to cleaving the viral polyprotein, PL^{pro} and the equivalent PLP2 have acquired DUB and deISGylase activity (65–73). Structure-guided mutagenesis of PL^{pro} of MERS-CoV and PLP2 of equine arterivirus (EAV) allowed the DUB activity to be separated from the proteolytic activity portrayed towards the viral polyprotein (72, 73). This uncoupling of these two different proteolytic activities indicated that the DUB activity of PL^{pro}/PLP2 contributes to the suppression of IFN-α/β transcription (72, 73). Unfortunately, it has not been determined whether PL^{pro}/PLP2 cleaves RLR signaling proteins and thus it is unclear what other proteolytic activities could be affected by the mutations that were introduced. Notably, the cleavage site of nidovirus PL^{pro}/PLP2 and FMDV L^{pro} in ubiquitin and ISG15 is different. While SARS-CoV PL^{pro} breaks the iso-peptide bond between ubiquitin or ISG15 and the target protein (65, 66), FMDV L^{pro} is a non-canonical deISGylase that targets a peptide bond in ISG15 itself, resulting in a diglycylated-lysine in the target protein (40). In conclusion, nidovirus PL^{pro}/PLP2 and FMDV L^{pro} are both papain-like proteases, but they have evolved different strategies to suppress IFN-α/β gene transcription.

FMDV L^{pro} and enterovirus 2A^{pro} are structurally different enzymes that share many functions; both cleave translation initiation factor eIF4G (34, 74, 75), both reduce IFN-α/β gene transcription (27, 47, 76, 77), both have been implicated in the suppression of SG formation (77–79) and both have been suggested to rescue viral translation from the

inhibitory effects of p-eIF2 (80, 81). Importantly, L^{pro} and 2A^{pro} both cleave several RLR signaling proteins, but the only overlapping RLR protein is MAVS (27, 76). Although a causal relationship between cleavage of RLR proteins and suppression of IFN- α/β transcription remains to be established for both proteases, the convergence on the cleavage of MAVS is noteworthy. In the absence of sequence homology, no evolutionary basis for the functional similarities between the two picornavirus proteases can be determined. Possibly, the extensive similarities between FMDV L^{pro} and enterovirus 2A^{pro}, both picornavirus proteases, is illustrative of the urgency for picornaviruses to suppress these particular antiviral host responses.

Our data suggests that the deISGylase activity of L^{pro} is not critically needed to suppress RLR signaling, but rather its role should be sought in the broader antiviral activities of ISG15 (reviewed in (10, 11)). It should be noted that our experiments were performed in naïve cells at high multiplicity of infection. Expression of the ISGylation machinery as well as ISG15 itself are boosted by IFN- α/β and therefore we cannot formally exclude a role for the DUB and/or deISGylase activity of L^{pro} in suppressing RLR signaling under different conditions (e.g. in IFN-primed cells). ISG15 has many functions, both intracellular and extracellular. Intracellular ISG15 can act both pro-inflammatory and immunomodulatory, either via ISGylation of target proteins or as free ISG15. Moreover, ISG15 can be secreted to act as a cytokine (25, 26). ISG15 also plays a role in damage repair after clearing viral infection (82) and can regulate cellular processes such as autophagy and metabolism (24, 83–85). How the deISGylase activity of L^{pro} contributes to efficient *in vivo* infection, remains to be established.

Materials and Methods

Cells and viruses. HeLa R19, HeLa R19 TBK1 k.o. and Hek293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS (V/V). HeLa OHIO cells were maintained in DMEM supplemented with 10% FCS (V/V) and 1% penicillin/streptomycin. LFPK $\alpha\beta$ 6 cells (86) were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the PIADC. These cells were maintained in minimal essential medium (MEM) supplemented with 10% FCS (V/V) and 1% antibiotics and non-essential amino acids. BHK-21 cells used for FMDV propagation were maintained in MEM supplemented with 10% FCS (V/V), 10% tryptose phosphate broth, 1% antibiotics and non-essential amino acids. HeLa R19 TBK1 k.o. cells were generated via CRISPR/cas9 methodology using a pCRISPR plasmid, as described previously (87). The used gRNA sequences are 5'-GCTACTGCAAATGTCTTTCG-3' and 5'-GAGGAAAACAGATTGGTT-3'. FMDV A12-WT (wild type) was generated from the full-length serotype A12 infectious clone,

pRMC35 (88) and A12-LLV2 (leaderless virus) was derived from the infectious clone lacking the Lb coding region, pRM-LLV2 (89). Viruses were propagated in BHK-21 and concentrated by polyethylene glycol precipitation, titrated on BHK-21 cells, and stored at -70°C. ERAV (NM-11/67 strain) (gift from D. Rowlands and T. Tuthill (90)) was obtained after passage on HeLa R19 cells and subsequently concentrated by ultracentrifugation through a 30% sucrose cushion at 140,000xg for 16 hours in a SW32Ti rotor and stored at -80°C. Recombinant EMCVs were generated by cloning the genes of interest into the XhoI/NotI restriction sites from the pM16.1-VFETQG-Zn infectious clone that was described previously (45). EMCV-L^{pro} viruses were recovered by transfection of run-off RNA transcripts into BHK-21 cells. Upon total CPE, viruses were concentrated by ultracentrifugation (as described for ERAV) and stored at -80°C.

Antibodies. The following antibodies were used for Western blot staining procedures: α FMDV VP1 (rabbit polyclonal Abmade at PIADC), α EMCV capsid (gift from Ann Palmenberg), α L^{pro} (gift from Ewald Beck and Tim Skern), α MAVS (Enzo life science ALX-210-929), α TBK1 (Cell signaling 3504), α IRF3 (Santa cruz sc-9082), α NF- κ B-p65/RelA (Santa Cruz Biotechnology SC-8008), α eIF4G (Bethyl laboratories A300-502A), α G3BP1 (BD biosciences clone 23/G3BP), α PARP (Roche Diagnostics #11835238001), α Flag (Sigma M2), α GFP (Invitrogen OSE00003G), α His (GE Healthcare, 27-4710-01), α Myc (Clone 4A6, Millipore), α HA (Abcam ab130275) and α tubulin (Sigma DM1A). Respective IRdye680 or IRdye800 conjugated secondary antibodies (LiCOR) or HRP-conjugated secondary antibodies were used for detection.

RT-PCR analysis. HeLa R19 cells were seeded in 24-wells plates and the next day infected with the indicated viruses at MOI 10 or transfected with the indicated plasmids or vRNA. Plasmids were transfected using Eugene6 (Promega) and vRNA was transfected using Lipofectamine 2000 (Invitrogen), both according to the manufacturer's instructions. Preparation of viral dsRNA and the pcDNA-GFP-MAVS construct have been described previously (1, 44). pEGFP-IRF3 [D5] construct was a kind gift from John Hiscott (91). At the indicated time points cells were lysed and cellular RNA was isolated using total RNA isolation kit (Machery-Nagel) according to manufacturer's instructions. Reverse transcription was set up using TaqMan Reverse Transcription Reagents (Applied Biosystems) before performing qPCR analysis with SYBR green (Roche) as described previously (92).

Western Blot analysis of transfected cells. The pIRES-EGFP-FMDV L^{pro} plasmid was described previously (45). The pcDNA-Flag-TBK1 plasmid was a gift from John Hiscott (93) and the pEF-FLAG-RIG-I was a gift from Takashi Fujita (94). HA-ubiquitin was expressed from a pCMV5 plasmid. Hek293T cells were seeded in 6-well plates and the next day transfected with 1.5 μ g of total plasmid using Eugene6 (Promega) according to

manufacturer's instructions. 16h posttransfection cells were lysed 100 µl lysisbuffer (100 mM Tris pH 8.0, 1 mM EDTA, 50 mM NaCl, 1% NP40, protease inhibitor mix (Roche)). Post nuclear lysate was obtained by centrifugation at 15000xg at 4 °C for 15 min. The amount of total protein in the lysates was determined using BCA assay (ThermoFisher) and 100 µg of protein was resolved using reducing sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.2 µm nitrocellulose membranes by wet electrophoretic transfer. Membranes were incubated 1h in blocking buffer (PBS + 0.1% Tween 20 + 2% BSA) and successively incubated overnight with primary antibodies diluted in blocking buffer and then for 30 min with respective secondary antibodies diluted in blocking buffer. Between and after the incubations, the membranes were washed three times with PBS+0.1% Tween-20. Finally, membranes were washed once with PBS and scanned using an Odyssey Imager (Li-COR).

Western Blot analysis of infected cells. HeLa R19 cells were seeded in 10 cm dishes and infected the next day with the indicated viruses at MOI 10. At the indicated time points cells were released using trypsin, washed once in PBS and lysed in 100 µl lysis buffer (100 mM Tris pH 8.0, 1 mM EDTA, 50 mM NaCl, 1% NP40, protease inhibitor mix (Roche)). Subsequent steps are identical as described for transfected cells. For the analysis of FMDV-infected LFBK αvβ6 cultures, cells were lysed in lysis buffer (0.5% NP-40 substitute, 50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA). Lysates were incubated at 4°C for 10 min and cellular debris was collected by centrifugation at 10,000xg for 15 min at 4°C. 40 ng of protein was resolved by SDS-PAGE, transferred by Western blot and secondary antibodies conjugated with horseradish peroxidase (Pierce) were used for detection of proteins. Following incubation with appropriate primary and secondary antibodies, protein bands were visualized using SuperSignal® West Dura Extended Duration Substrate (ThermoScientific, Rockford, IL, USA) according to the manufacturer's directions.

***In vitro* TBK1 cleavage.** sL^{pro} was expressed and purified as reported previously (95). 275 ng of His-hTBK1 (Millipore) was incubated with 0-3 µg sL^{pro} for 2 h at 30 °C in a HEPES buffer (20 mM HEPES pH 7.4, 150 mM KCl, 1 mM EDTA) before the reaction mixture was dissolved on SDS-PAGE. Proteins were transferred to nitrocellulose and Western blot staining for the his-tag was performed. Myc-mTBK1 and Myc-mTBK1_{692AAA694} were transiently expressed in HeLa OHIO cells from plasmid pCS2-6Myc-mTBK1, a gift from T. Decker. 20 µg myc-tagged mTBK1 containing cell lysate was incubated with 2 µg sL^{pro} for 2 h at 30 °C in a HEPES buffer before resolving the reaction mixture on SDS-PAGE, transferring the protein to nitrocellulose membrane and performing Western blot staining for Myc.

***In vitro* DUB and deISGylase assays.** Ubiquitin/ISG15-TAMRA assays were performed according to (96). Di-ubiquitin *in vitro* cleavage assays were performed as described previously (97).

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Supplementary Information

Figure S1: N-terminal TBK1 cleavage fragment can facilitate RLR signaling. (A) HeLa R19 TBK1 k.o. cells were generated using CRISPR/cas9 technology. Wt and TBK1 k.o. cells were lysed and lysates subjected to Western Blot analysis for TBK1 and tubulin. (B) IFN-β induction upon various triggers of RLR signaling was compared in wt and TBK1 k.o. cells. Cells were infected with EMCV-L^{zn} virus at MOI 10, transfected with 20 ng vRNA, or transfected with 1 μg of plasmid expressing MAVS or IRF3. Cells were lysed at 8 h pi or transfection. Total RNA was isolated and used for RT-qPCR analysis for IFN-β and actin mRNA. The IFN-β levels are depicted as a fold induction compared to levels in mock-treated cells, after correction for actin mRNA levels. Error bars depict the SD. (C) HeLa R19 TBK1 k.o. cells were transfected with 2 μg plasmid expressing full-length or truncated TBK1 (TBK1 Δ35aa). TBK1 Δ35aa is representative for the L^{pro}-generated N-terminal cleavage product. Cells were lysed and lysates subjected to Western Blot analysis for TBK1 and tubulin. (D) TBK1 k.o. cells were reconstituted with full-length TBK1 as described for (C) and subsequently transfected with 100 ng poly(I:C). Cells were lysed at 8 h post transfection of poly(I:C). Total RNA was isolated and used for RT-qPCR analysis for IFN-β and actin mRNA. The IFN-β levels are depicted as a fold induction compared to levels in mock-treated cells, after correction for actin mRNA levels. Error bars depict the SD. (E) TBK1 k.o. cells were reconstituted with full-length or truncated TBK1 (TBK1 Δ35aa) as described for (C). Subsequent steps as described for (D). Error bars depict the SD.

Chapter 5

Essential role of enterovirus 2A protease in counteracting stress granule formation and the induction of type I interferon

5

Linda J. Visser¹, Martijn A. Langereis¹, Huib H. Rabouw¹, Maryam Wahedi¹, Elke M. Muntjewerff¹, Raoul J. de Groot¹, Frank J.M. van Kuppeveld^{1#}

¹Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

[#]Corresponding author.

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Abstract

Most viruses have acquired mechanisms to suppress antiviral IFN- α/β and stress responses. Enteroviruses actively counteract the induction of IFN- α/β gene transcription and stress granule (SG) formation, which are increasingly implicated as a platform for antiviral signaling, but the underlying mechanisms remain poorly understood. Both viral proteases (2A^{pro} and 3C^{pro}) have been implicated to suppress these responses, but these conclusions predominantly rely on ectopic overexpression of viral proteases or addition of purified viral proteases to cell lysates. Here, we present a detailed and comprehensive comparison of the effect of individual enterovirus proteases on the formation of SGs and the induction of IFN- α/β gene expression in infected cells, for representative members of the enterovirus species EV-A to EV-D. First, we show that SG formation and IFN- β induction are suppressed in cells infected with EV-A71, CV-B3, CV-A21 and EV-D68. By introducing genes encoding CV-B3 proteases in a recombinant encephalomyocarditis virus (EMCV) that was designed to efficiently activate antiviral responses, we show that CV-B3 2A^{pro}, but not 3C^{pro}, is the major antagonist that counters SG formation and IFN- β gene transcription, and that 2A^{pro}'s proteolytic activity is essential for both functions. 2A^{pro} efficiently suppressed SG formation despite PKR activation and eIF2 α phosphorylation, suggesting that 2A^{pro} antagonizes SG assembly or promotes their disassembly. Finally, we show that the ability to suppress SG formation and IFN- β gene transcription is conserved in the 2A^{pro} of EV-A71, CV-A21 and EV-D68. Collectively, our results indicate that enterovirus 2A^{pro} plays a key role in inhibiting innate antiviral cellular responses.

Importance

Enteroviruses are important pathogens that can cause a variety of diseases in humans, including aseptic meningitis, myocarditis, hand-foot-and-mouth disease, conjunctivitis and acute flaccid paralysis. Like many other viruses, enteroviruses must counteract antiviral cellular responses to establish an infection. It has been suggested that enterovirus proteases cleave cellular factors to perturb antiviral pathways, but the exact contribution of viral proteases 2A^{pro} and 3C^{pro} remains elusive. Here, we show that 2A^{pro}, but not 3C^{pro}, of all four human EV species (EV-A to EV-D) inhibits SG formation and IFN- β gene transcription. Our observations suggest that enterovirus 2A^{pro} has a conserved function in counteracting antiviral host responses, and thereby is the main enterovirus “security protein”. Understanding the molecular mechanisms of enterovirus immune evasion strategies may help to develop countermeasures to control infections with these viruses.

Introduction

Picornaviruses are a large and diverse family of small (~30 nm) non-enveloped viruses with a positive-sense RNA genome. Especially the genus *Enterovirus* encompasses many human pathogens with implications for public health. The enteroviruses are categorized into fifteen enterovirus species (EV-A to EV-L) and three rhinovirus species (RV-A to RV-C). Human pathogens are found amongst the EV-A to EV-D and the three rhinovirus species. The best-known pathogenic enterovirus is poliovirus (PV), the causative agent of poliomyelitis. In addition, coxsackie A and B viruses, echoviruses and several numbered enteroviruses (e.g. EV-A71 and EV-D68) cause a broad range of diseases, such as aseptic meningitis, myocarditis, hand-foot-and-mouth disease, conjunctivitis and acute flaccid paralysis. Rhinoviruses are the leading cause of the common cold, and are associated with exacerbations of asthma and chronic obstructive pulmonary disease (COPD) (reviewed in (1)). The enterovirus genome is translated into one polyprotein that is autocatalytically processed into the structural proteins (VP1, VP2, VP3, VP4), the non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D) and several cleavage intermediates (e.g. 3CD) (reviewed in (1)). While the structural proteins make up the viral capsid, the non-structural proteins are involved in virus replication and/or interfere with antiviral responses. Non-structural proteins 2A and 3C are the proteases involved in processing of the viral polyprotein. While 3C^{pro} is responsible for the majority of the cleavages needed for polyprotein processing, 2A^{pro} cleaves at the junction between the structural proteins (P1 region) and its own N-terminus (reviewed in (1)). Besides their essential role in polyprotein processing, both proteases also cleave host cell factors to optimize virus replication. For example, 2A^{pro} mediates host shutdown by cleaving translation initiation factor 4G (eIF4G) (2).

In response to virus infections, cells activate several antiviral responses. A well-known antiviral response is the induction of type I interferons (IFN- α/β). Viral genome replication generates double-stranded RNA (dsRNA) replication intermediates, which can be recognized by cytoplasmic RIG-I like receptors (RLRs). Enterovirus replication intermediates are predominantly recognized by MDA5(3–5). However, recent evidence suggests that RIG-I may also contribute to the induction of IFN- α/β in certain cell types (6). Upon recognition of viral dsRNA, MDA5 interacts with MAVS, which subsequently activates TRAF3, and TBK1. TBK1 phosphorylates the transcription factors IRF3 and IRF7, resulting in their activation and dimerization. Simultaneously, TRAF3 activates the transcription factor NF κ B. Upon activation, IRF3, IRF7 and NF κ B localize to the nucleus, where they induce expression of IFN- α/β and other proinflammatory cytokines. Subsequent IFN- α/β signaling via the type I IFN receptor (IFNAR) and the JAK-STAT pathway induces the expression of hundreds of interferon stimulated genes (ISGs). IFN- α/β signals in autocrine and paracrine ways to induce a tissue-wide antiviral state, thereby limiting viral spread (reviewed in (7, 8)).

One of the ISGs is dsRNA-dependent protein kinase R (PKR), a sensor protein of the Integrated Stress Response (ISR). When PKR binds dsRNA ligands, it dimerizes, autophosphorylates, and subsequently phosphorylates the α -subunit of translation initiation factor 2 (eIF2 α). Phosphorylated eIF2 α (p-eIF2 α) represses translation and thereby impairs virus propagation. Stalled messenger ribonucleoproteins (mRNPs) – consisting of mRNA, small ribosomal subunits, translation initiation factors (eIFs), and several auxiliary proteins – are stored in cytoplasmic aggregates known as stress granules (SG). SG formation is directed by SG scaffolding proteins, such as GTPase-activating protein-binding proteins 1 and 2 (G3BP1 and G3BP2) and T cell-restricted intracellular antigen 1 (TIA-1) (reviewed in (9)). Rather than merely being an outcome of translation inhibition, SGs have also been suggested to serve as a platform for antiviral signaling. Multiple signaling molecules – including MDA5, RIG-I, PKR and TRAF2 – are recruited to SGs and this localization has been suggested to regulate their activity (10–16). Further evidence for an antiviral function of SGs is the growing evidence that many viruses – apart from their effect on the upstream signaling cascade – have evolved mechanisms to counteract SG formation by targeting SG components (15, 17–20). The exact function of SGs, however, remains to be established.

To establish a productive infection, enteroviruses actively suppress antiviral responses. Several studies have shown that enteroviruses suppress IRF3 dimerization and thereby IFN- α/β gene transcription (11, 21–25). Both viral proteases, 2A^{pro} and 3C^{pro}, cleave various factors of the RLR signaling pathway, implicating them in the suppression of IFN- α/β gene transcription. Overexpression of 3C^{pro} of several enteroviruses has been shown to result in the cleavage of MAVS, IRF7 and IRF9 and - by consequence - in the suppression of a co-expressed IFN reporter construct (24–28). However, in several of these studies, convincing evidence that the cleavage products observed upon 3C^{pro} overexpression are the same as in enterovirus-infected cells is lacking. In fact, we and others have identified 2A^{pro} as the viral protease responsible for cleaving MAVS (21, 22). Addition of recombinant 2A^{pro}, but not 3C^{pro}, to cell lysates resulted in the appearance of MAVS cleavage products of the same size as observed in infected cells. These cleavage products were also observed when 2A^{pro}, but not 3C^{pro}, was expressed by a recombinant EMCV, a picornavirus that by itself does not cleave components of the RLR pathway (21). Besides cleaving MAVS, 2A^{pro} has also been suggested to cleave MDA5 (21), which contradicted earlier suggestions that MDA5 is degraded by the proteasome (29). Meanwhile, 3C^{pro} cleaves RIG-I (21, 30). Contradictory results have also been reported on the mechanism how enteroviruses interfere with IFN- α/β signaling. Some studies reported that EV-A71 infection reduces the expression levels of the IFN receptor, blocks STAT1 and STAT2 phosphorylation, and suppresses expression of multiple ISGs, through the action of 2A^{pro} (31, 32). In another study, however, it was suggested that EV-A71 infection does not affect STAT1 and STAT2 phosphorylation, but that 3C^{pro} prevents

these molecules from entering the nucleus (33). Overall, these conflicting data indicate that the physiological role of the 2A^{pro}- and/or 3C^{pro}-mediated cleavages of signaling molecules for the suppression of the RLR and JAK-STAT pathways requires further investigation.

Enteroviruses also actively suppress the ISR. Infection of cells with PV, CV-B3 and EV-A71 results in the formation of small SGs early in infection, but these disappear later in infection (34–37). Again, conflicting observations about the identity of the viral antagonist have been reported. Initially, the suppression of SGs was attributed to 3C^{pro} (34–36). 3C^{pro} was shown to cleave G3BP1 and suppress SG formation, and overexpression of a cleavage-resistant G3BP1 decreased virus replication, although this decrease was rather modest (34, 36, 38). Recently, however, evidence was presented that 2A^{pro} from EV-A71, PV or CV suppresses SG formation induced by infection, as well as upon induction by sodium arsenite or heat shock (37).

In this study, we set out to directly compare the abilities of 2A^{pro} and 3C^{pro} of multiple enteroviruses to suppress IFN- α/β induction and SG formation, in infected cells. As described above, we previously introduced the genes encoding 2A^{pro} and 3C^{pro} of several enteroviruses in EMCV to study 2A^{pro}- and 3C^{pro}-mediated cleavages in infected cells (21). However, these recombinant EMCVs contained an intact antagonist of the antiviral responses, i.e. the leader (L) protein, and therefore these viruses could not be used to study the effect of the enterovirus proteases on IFN- α/β mRNA levels and SG formation. To overcome this, we introduced the genes encoding the enterovirus proteases in a mutant EMCV containing mutations that inactivate the leader protein (EMCV-L^{zn}) (3, 11, 39). Using these recombinant viruses, we provide a comprehensive and detailed analysis of the effect of the proteases of representative members of the enterovirus species EV-A to EV-D on the formation of SGs and the induction of IFN- α/β . We demonstrate that 2A^{pro}, not 3C^{pro}, of EV-A71, CV-B3, CV-A21 and EV-D68 strongly suppresses IFN- α/β induction and SG formation in virus-infected cells, thereby pinpointing 2A^{pro} as a major antagonist of these antiviral responses.

Results

Multiple enteroviruses suppress SG formation. We first determined whether representative members of the enterovirus species EV-A – EV-D share the ability to suppress SGs. To this end, we infected HeLa cells with EV-A71, CV-B3, CV-A21 or EV-D68 and visualized SGs. We also infected cells with EMCV-L^{zn}, which induces SG formation (11) and therefore served as a positive control. At 4 h and 6 h post infection (hpi) cells were fixed and we performed immunofluorescence staining for dsRNA, to visualize viral replication, and the SG markers

G3BP1 and eIF3. Infection with all the viruses led to an increase in dsRNA signal, indicating that all viruses replicated efficiently. In the cells infected with the different enteroviruses we observed small SGs at 4 hpi (Fig 1). These SGs were no longer detected at 6 hpi in cells infected with CV-B3 or CV-A21, while the majority of the cells infected with EV-A71 and EV-D68 also lacked SGs at 6 hpi (Fig 1). Suppression of SG formation was previously reported for PV (34), CV-B3 (35) and – while this work was in progress – also for EV-A71 (36, 37). Here, we provide evidence that also CV-A21, another member of the EV-C species, and EV-D68, an EV-D species member, suppress the formation of SGs. Taken together, our data suggest that the ability to counteract SG formation is conserved amongst all human enterovirus species (i.e. EV-A to EV-D).

Overexpression of enterovirus 2A^{pro} suppresses sodium arsenite-induced SGs. To better understand the effects of enterovirus proteases on SG formation, we overexpressed 2A^{pro} and 3C^{pro} from EV-A71, CV-B3 or PV as EGFP fusion proteins and visualized SGs in the transfected cells. The cells were treated with sodium arsenite to induce SG formation. Afterwards we performed immunofluorescence staining against G3BP1 and TIA-1 (Fig. 2). In our hands, enterovirus 2A^{pro} inhibited SG formation (Fig. 2A). In cells expressing EGFP-2A^{pro} we did not detect any G3BP1 positive SGs. However, we did observe a few TIA-1 granules. Meanwhile, expression of EGFP-3C^{pro} had no effect on SG formation (Fig. 2B). These findings suggest that enterovirus 2A^{pro} is the major antagonist of SG formation.

Enterovirus 2A^{pro} suppresses SG formation when introduced into recombinant EMCV containing an inactive stress antagonist. Overexpression of enteroviral proteases, followed by sodium arsenite treatment to induce SGs, may not accurately represent the role of the proteases in an enterovirus-infected cell. Given the essential role of the viral proteases in polyprotein processing and RNA replication, it is difficult to study their role in suppressing SG formation by introducing proteolytically inactivating mutations in the viral genome. We previously generated recombinant EMCVs harboring 2A^{pro} and 3C^{pro} of different enteroviruses upstream of the leader protein, to study the proteolytic activity of 2A^{pro} and 3C^{pro} (21). Unfortunately, we could not use these viruses to study the effects of the enterovirus proteases on SG formation as EMCV actively suppresses SG formation. It has been suggested that EMCV 3C^{pro} cleaves G3BP1 and that this prevents SG assembly, although this cleavage is observed only at very late stages of infection (10-12 hpi) (40). In contrast, we and others did not observe G3BP1 cleavage in cells infected with either EMCV or TMEV, and instead provided extensive evidence that the leader protein has an essential role in suppressing SG formation in cardiovirus-infected cells (11, 41, 42). As part of these studies, we generated EMCV-L^{Zn}, a mutant virus in which the leader protein is inactivated by mutations that disrupt its zinc finger domain and that, by consequence, can no longer suppress SG formation. To investigate the role of enterovirus proteases in suppressing SG

Figure 1: Multiple enteroviruses suppress SG formation. HeLa R19 cells were infected with EV-A71, CV-B3, CV-A21, EV-D68 or EMCV-L^{Zn} at MOI 10 and cells were fixed at 4 or 6 hpi. Subsequently, immunofluorescence staining was performed for the SG markers eIF3 and G3BP2, and viral replication was visualized by dsRNA staining.

formation, we therefore inserted the genes encoding CV-B3 2A^{pro} (EMCV-2A^{pro}) or CV-B3 3C^{pro} (EMCV-3C^{pro}) in EMCV-L^{Zn} (Fig 3A). In parallel, we generated EMCV-L^{Zn} viruses encoding catalytically inactive mutants of the CV-B3 2A^{pro} (EMCV-2Am, mutation C109A) and 3C^{pro} (EMCV-3Cm, mutation C147A), allowing us to determine whether the effects of 2A^{pro} and 3C^{pro} depend on their proteolytic activity. To ensure that CV-B3 2A^{pro} and 3C^{pro} are active in the context of EMCV-L^{Zn} infection, we infected HeLa cells with the different chimeric EMCVs and performed western blot analysis of eIF4G, a well-known 2A^{pro} target, or G3BP1, a well-known 3C^{pro} target (Fig 3B). Indeed, infection with EMCV-2A^{pro}, but not EMCV-2Am, resulted in cleavage of eIF4G, while infection with EMCV-3C^{pro} resulted in G3BP1 cleavage. No G3BP1 cleavage was observed in cells infected with either EMCV-L^{Zn}, which is consistent with our previous observations (11, 41, 42), or EMCV-3Cm.

Figure 2: Overexpression of enterovirus 2A^{pro} suppresses sodium arsenite-induced SGs. HeLa R19 cells were transfected with plasmids encoding the 2A^{pro} or 3C^{pro} of CV-B3, EV-A71 or PV, N-terminally fused to eGFP. 16 h post transfection, cells were incubated with 500 μ M sodium arsenite for 30 min. Subsequently, immunofluorescence staining was performed for the SG markers G3BP1 and TIA-1.

Next, we determined the ability of EMCV-2A^{pro} and EMCV-3C^{pro} to suppress SG formation. We infected HeLa cells with the different chimeric EMCVs, fixed the cells at 4 or 8 hpi and performed immunofluorescence staining for the SG markers eIF3 and G3BP2, and dsRNA to identify the infected cells (Fig 3C). Infection with EMCV-2A^{pro} resulted in the formation of some small SGs at 4 hpi. Importantly, we did not observe these small SGs upon infection

with the other chimeric EMCVs. This is consistent with previous reports that overexpression of 2A^{pro} can induce formation of some small SGs (31, 37, 38), most likely through its activity to cleave eIF4G and cause a host mRNA translation shut-off. Intriguingly, as infection with EMCV-2A^{pro} progressed, these small SGs disappeared and we did not observe any SGs at 8 hpi. A similar pattern of SGs that first appear and subsequently disappear during the course of infection has frequently been reported for enteroviruses (34–37). Infection with EMCV-2Am did not induce small SGs at 4 hpi, nor did it suppress formation of larger SGs at 8 hpi. This indicates that the catalytic activity of 2A^{pro} is needed for both the induction of small SGs at 4 hpi and the subsequent suppression of the formation of larger SGs. In contrast to 2A^{pro} expression (EMCV-2A^{pro}), heterologous expression of 3C^{pro} (EMCV-3C^{pro}) had a small effect on SG formation. These data suggest that CVB3 2A^{pro}, rather than CVB3 3C^{pro}, is the main antagonist of SG formation.

To test whether 2A^{pro} is also the main stress antagonist of enteroviruses belonging to other species, we constructed EMCV-L^{Zn} viruses expressing either 2A^{pro} or 3C^{pro} from EV-A71, CV-A21, and EV-D68, and determined whether these chimeric viruses were able to suppress SG formation. HeLa cells were infected with these recombinant EMCV viruses and we visualized SGs by immunofluorescence staining. Subsequently, we quantified the number of SGs per cell (Fig. 3D). The different 2A^{pro}-expressing viruses all efficiently reduced the number of SGs (~90%), whereas infection with the different 3C^{pro}-expressing viruses had a less pronounced effect on the number of SGs (~25-40% reduction). Overall, our data demonstrate that both enterovirus proteases have an effect on SG formation in HeLa cells, but that 2A^{pro} is the main antagonist of SG formation and that this function is conserved among enteroviruses belonging to species EV-A to EV-D.

Expression of 2A^{pro} restores the impaired replication of EMCV-L^{Zn}. EMCV-L^{Zn} replication in HeLa cells is predominantly restricted by the ISR (43). We next asked whether heterologous expression of enterovirus 2A^{pro}, which efficiently suppressed SG formation, restored replication of EMCV-L^{Zn}. To investigate this, we compared the replication kinetics of EMCV-L^{Zn}, EMCV-2A^{pro} and EMCV-3C^{pro} (encoding the CV-B3 proteases) in a multi-cycle infection. We infected HeLa cells - wt or deficient in PKR (PKR k.o.) - at a low multiplicity of infection and determined virus yields at 24 hpi (Fig. 3E). In agreement with previous results (39, 43), we observed a strong defect in replication of EMCV-L^{Zn} in wt cells, but not in PKR k.o. cells, confirming that replication in HeLa cells is predominantly restricted by the ISR. EMCV-2A^{pro} replicated with similar efficiency in both cell lines, illustrating that heterologous expression of 2A^{pro} rescued EMCV-L^{Zn} replication by countering the antiviral function of PKR-activated ISR. In contrast, expression of 3C^{pro} failed to rescue EMCV-L^{Zn} replication. Collectively, these data point to an important antagonistic function of 2A^{pro} in counteracting PKR-induced SG formation in virus infected cells.

< Figure 3: Enterovirus 2A^{pro} suppresses SG formation in EMCV-infected cells. (A) Schematic representation of EMCV-L^{Zn} genome encoding 2A^{pro} or 3C^{pro}. The endogenous stress response antagonist (Leader) has been inactivated by point mutations in its Zn-finger domain (C19A/C22A), and subsequently the genes encoding 2A^{pro} and 3C^{pro} were introduced at the 5' end of the EMCV open reading frame. (B) HeLa R19 cells were infected with EMCV-L^{Zn}, EMCV-2A^{pro}, EMCV-2Am, EMCV-3C^{pro} or EMCV-3Cm at MOI 10 and cells were lysed at 8 hpi. Lysates were subjected to western blot analysis for eIF4G and G3BP1, and tubulin served as a loading control. (C) HeLa R19 cells were infected with EMCV-L^{Zn}, EMCV-2A^{pro}, EMCV-2Am, EMCV-3C^{pro} or EMCV-3Cm at MOI 10 and cells were fixed at 4 or 8 hpi. Subsequently immunofluorescence staining was performed for the SG markers eIF3 and G3BP2. Viral replication was visualized by dsRNA staining. (D) HeLa R19 cells were infected with EMCV-L^{Zn} encoding the 2A^{pro} or 3C^{pro} of EV-A71, CV-B3, CV-A21 or EV-D68 at MOI 10 and fixed at 8 hpi. Subsequently immunofluorescence staining was performed for G3BP1 and the number of SGs/cell was analyzed for at least 50 cells per condition. (E) HeLa R19 cells, wt or deficient in PKR expression, were infected at MOI 0.01 with EMCV, EMCV-L^{Zn}, EMCV-2A^{pro} or EMCV-3C^{pro}. At 24 hpi, samples were frozen and subjected to 3 freeze-thaw cycles. Subsequently, viral yield was determined by endpoint dilution on BHK21 cells.

Figure 4: Enterovirus 2A^{pro} does not affect PKR signaling. HeLa R19 cells were infected with EMCV-L^{Zn}, EMCV-2A^{pro} or EMCV-2Am at MOI 10 and fixed at 8 hpi. Subsequently, cells were stained with antibodies against dsRNA and p-PKR or dsRNA and p-eIF2α for flowcytometric analysis. Graphs represent the level of p-PKR or p-eIF2α in dsRNA positive (infected) cells.

Enterovirus 2A^{pro} does not affect PKR signaling. 2A^{pro} depends on its catalytic activity to suppress SGs, which suggests that 2A^{pro} cleaves a cellular factor to suppress SGs. 2A^{pro} does not cleave known structural SG components like G3BP1, G3BP2 or TIA-1 (35, 37, 38). To investigate whether 2A^{pro} affects PKR signaling, we infected HeLa cells with EMCV-L^{Zn}, EMCV-2A^{pro} or EMCV-2Am and determined the levels of p-PKR and p-eIF2α at 8 hpi by flow cytometry (Fig 4). Infection with EMCV-L^{Zn} activated the PKR signaling pathway, resulting in an increased level of p-PKR and p-eIF2α relative to mock infected cells, consistent with previous results (43). Infection with EMCV-2A^{pro} and EMCV-2Am resulted in similar increases in p-PKR and p-eIF2α levels, demonstrating that 2A^{pro} does not affect the PKR signaling pathway. Thus, it is unlikely that 2A^{pro} suppresses SG formation by cleaving a factor involved in PKR signaling. How 2A^{pro} suppresses SG formation remains to be established.

Enterovirus 2A^{pro} suppresses the induction of type I IFN gene transcription. In addition to the ISR, enteroviruses are known to suppress the induction of type I IFNs (11, 21–25). Indeed, infection of HeLa cells with EV-A71, CV-B3, CV-A21 or PV did not significantly induce expression of IFN- β mRNA at 8 hr p.i. (Fig. 5A). Various studies have shown that both 2A^{pro} and 3C^{pro} can cleave proteins in the RLR signaling pathway, and thus may be involved in suppressing IFN- β gene transcription in infected cells (21–27, 30, 44, 45). To evaluate the effect of the CV-B3 proteases on IFN- β mRNA induction in infected cells, we infected HeLa cells with EMCV-2A^{pro} or EMCV-3C^{pro} and determined the induction of IFN- β mRNA at 8 hpi by RT-qPCR analysis (Fig. 5B). As described previously (3, 11, 39), EMCV-L^{Zn} failed to suppress the RLR signaling pathway and resulted in high IFN- β mRNA levels. In contrast, infection with EMCV-2A^{pro} hardly induced IFN- β mRNA levels, while infection with EMCV-2Am resulted in high IFN- β mRNA levels, indicating that 2A^{pro} - via its catalytic activity - suppresses IFN- β gene transcription. Infection with EMCV-3C^{pro} resulted in high levels of IFN- β mRNA, indicating that 3C^{pro} is unable to suppress RLR-mediated signaling in HeLa cells. Collectively, these data suggest that enterovirus 2A^{pro} is the viral protease that is responsible for suppressing IFN- β gene transcription in infected HeLa cells. We also tested the effect of 2A^{pro} and 3C^{pro} on JAK-STAT signaling by investigating the expression levels of ISGs. We determined the induction of ISG56 and MDA5 gene transcription via RT-qPCR analysis (Fig 5C and D). Consistent with the ability of 2A^{pro} to suppress IFN- β gene transcription, we did not observe an increase in the expression of these ISGs. 3C^{pro} had no effect on the induction of IFN- β gene transcription (Fig. 5B), yet we observed a ~10-fold reduction in the expression of both ISG56 and MDA5, suggesting that 3C^{pro} affects JAK-STAT signaling rather than RLR signaling.

To determine whether the 2A^{pro} of other enteroviruses also suppress IFN- β gene transcription, we infected cells with EMCVs expressing 2A^{pro} or 3C^{pro} from EV-A71, CV-A21, and EV-D68 and determined IFN- β mRNA levels via RT-qPCR analysis (Fig. 5E). Indeed, 2A^{pro} of these different enteroviruses, suppressed IFN- β gene expression in the context of EMCV, while 3C^{pro} of these viruses did not. This demonstrates that the ability of 2A^{pro} to suppress the induction of IFN- β gene expression is conserved among EV-A to EV-D species members.

Inhibition of SG formation by 2A^{pro} unlikely accounts for the inhibition of type I IFN gene transcription. It has been suggested that SG formation is critical for the induction of type I IFN (10, 40). This raised the possibility that 2A^{pro} suppresses the induction of IFN- β mRNA via its ability to inhibit SG formation. To investigate the role of SGs in the induction of type I IFN we used PKR k.o. cells, which cannot form SGs. Two types of PKR k.o. MEFs have been described (46–48); one lacks the RNA binding domain (PKR RBD k.o.), while the other lacks the kinase domain (PKR KD k.o.) (Fig 6A). Interestingly, while it was shown that the PKR RBD k.o. cells fail to induce IFN expression (10), we previously observed only a 5- to 10-fold reduction in IFN β mRNA levels in PKR KD k.o. MEFs (11). To better understand these

Figure 5: Enterovirus 2A^{pro} suppresses the induction of type I IFN gene expression. (A) HeLa R19 cells were infected with the indicated viruses at MOI 10 and cells were lysed at 8 hpi, total cellular RNA was isolated and RT-qPCR analysis was performed for IFN- β and actin mRNA. (B, C, D) HeLa R19 cells were infected with CV-B3, EMCV, EMCV-L^{Zn}, EMCV-2A^{pro}, EMCV-2Am, EMCV-3C^{pro} or EMCV-3Cm at MOI 10 and cells were lysed at 8 hpi, total cellular RNA was isolated and RT-qPCR analysis was performed for IFN- β , ISG56, MDA5 and actin mRNA. (E) HeLa R19 cells were infected with EMCV-L^{Zn} encoding the 2A^{pro} or 3C^{pro} of EV-A71, CV-B3, CV-A21 or EV-D68 at MOI 10 and cells were lysed at 8 hpi, total cellular RNA was isolated and RT-qPCR analysis was performed for IFN- β and actin mRNA, as well as EMCV vRNA. All graphs depict the fold induction of target gene mRNA levels compared to mock infected cells, after correction for actin mRNA levels, with the exception of EMCV vRNA, which is shown as a copy number per cell, calculated from a plasmid standard. One-way ANOVA with Dunnet post-hoc test was used to determine statistical significance compared to EMCV (A-D) or EMCV-L^{Zn} (E) infected cells (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

different results, we compared both types of PKR k.o. MEFs side-by-side under identical experimental conditions. We confirmed that both cell lines express truncated PKR using Western blot analysis (Fig 6B), and that both have lost the ability to form SGs (Fig 6C). Upon infection with EMCV-L^{Zn}, the PKR RBD k.o. cells showed no IFN β mRNA induction, while the PKR KD k.o. cells showed only a relatively small reduction (5- to 10-fold) in IFN β mRNA levels. Why the two cell lines yield such different results remains to be elucidated. In addition to its role in the ISR, PKR has been suggested to play a role in the MDA5 signaling pathway (49). Possibly, the RBD is of greater importance for this function.

Figure 6: Two types of PKR k.o. MEFs are both deficient in SG formation but differ in IFN- β induction. (A) Schematic representation of PKR gene and the two different types of PKR k.o. MEFs used in this study. In the PKR KD k.o. cells a premature stop codon was introduced before the kinase domain. In the PKR RBD k.o. cells the initial start codon was removed, resulting in a PKR that lacks the RNA binding domain. (B) PKR western blot analysis of lysates of PKR k.o. MEFs and the corresponding wt MEFs. (C) PKR k.o. and wt MEFs were infected with EMCV-L^{Zn} at MOI 10, fixed at 8 hpi, and stained for SG marker G3BP1. (D) PKR k.o. and wt MEFs were infected with EMCV-L^{Zn} at MOI 10 and lysed at 6 or 10 hpi. Total RNA was isolated and subjected to RT-qPCR analysis for IFN- β , actin mRNA and EMCV vRNA. IFN- β mRNA is depicted as the fold induction of IFN- β mRNA levels compared to mock infected cells, after correction for actin mRNA levels. EMCV vRNA is shown as a copy number per cell, calculated from a plasmid standard. Two-way ANOVA with Bonferroni post-hoc test was used to calculate statistical significance (** p < 0.01, *** p < 0.001, **** p < 0.0001).

The results described here above did not provide a clear answer on the role of SGs in the induction of type I IFN. Therefore, we studied the role of SGs in IFN- β induction via an alternative, PKR-independent, approach. To interfere with SG formation, we generated HeLa cells lacking expression of SG scaffolding proteins G3BP1 and/or G3BP2 using CRISPR/

cas9 technology (Fig 7A). To characterize our newly generated cell lines, we determined whether they form SGs upon infection with EMCV-L^{Zn} or addition of sodium arsenite (Fig 7B). While deletion of G3BP1 had a small effect on SG formation, G3BP1 and G3BP2 need to be simultaneously depleted (G3BP1/2 k.o.) to prevent SG formation, consistent with a previous siRNA-based study (50). To determine whether the PKR signaling pathway was affected by knocking out G3BP1 and/or G3BP2, we infected the different k.o. cell lines and performed Western blot analysis for PKR and p-PKR (Fig 7C). Infection with EMCV-L^{Zn} resulted in the phosphorylation of PKR in all cell lines, indicating that the signaling pathway was not affected. Next, we determined the induction of IFN- β gene transcription upon infection with EMCV-L^{Zn} via RT-qPCR analysis (Fig 7D). Only in the G3BP1/2 k.o. cells, which cannot form SGs, we observed 5- to 10-fold lower IFN β mRNA levels at 6 and 8 hpi. This reduction is similar to what we previously observed in the PKR KD k.o. MEFs. Collectively our data suggest that, while SGs contribute to the induction of IFN- β mRNA, they do not play a critical role. This implies that it is unlikely that the nearly complete inhibition of type I IFN gene transcription by 2A^{pro} is due to its ability to suppress SG formation.

Discussion

To establish an infection, viruses must navigate an intricate network of antiviral host responses. Enteroviruses suppress both SG formation and the induction of IFN- α/β . Since both enterovirus 2A^{pro} and 3C^{pro} are essential for viral polyprotein processing and virus replication, it is challenging to study the ability of these proteins to suppress SGs during enterovirus infection by impairing their function. Therefore, most previous work relies on overexpression data of 2A^{pro} and 3C^{pro}. However, overexpression of a viral protease may not accurately mimic its function during infection (21) and this may explain the conflicting results that have been reported on the role of these proteases in antagonizing the ISR and the IFN- α/β pathway. In this study, we demonstrated that EV-A71, CV-B3, CV-A21, EV-D68 suppress SG formation and IFN- β gene transcription in HeLa cells. To study the role of 2A^{pro} and 3C^{pro} in suppressing SG formation and IFN- β gene transcription, we introduced the proteases of CV-B3 in a recombinant EMCV that was designed to efficiently activate antiviral responses (EMCV-L^{Zn}). We demonstrated that 2A^{pro} strongly inhibited SG formation and IFN- β gene transcription in infected cells, suggesting that the cleavages mediated by 2A^{pro} are more important for suppressing antiviral host responses than those mediated by 3C^{pro}. Using the same system, we showed that 2A^{pro} of EV-A71, CVA-21 and EV-D68 also suppressed SG formation and IFN- β gene transcription, indicating that the identity of the antagonist is conserved among enteroviruses belonging to species EV-A to EV-D.

< **Figure 7: SG formation-deficient HeLa G3BP1/2 k.o. cells show only a small impairment in IFN-β induction.**

(A) Lysates of HeLa G3BP1 k.o., G3BP2 k.o. and G3BP1/2 k.o. cells were subjected to western blot analysis for G3BP1, G3BP2 and tubulin. (B) HeLa wt, G3BP1 k.o., G3BP2 k.o. and G3BP1/2 k.o. cells were infected with EMCV-L^{Zn} at MOI 10 or incubated with 500 μM sodium arsenite. Cells were fixed at 8hpi or 30 min after addition of sodium arsenite, and SGs were visualized by immunofluorescence staining for G3BP1, eIF3 and G3BP2. (C) HeLa wt, G3BP1 k.o., G3BP2 k.o. and G3BP1/2 k.o. cells were infected with EMCV-L^{Zn} at MOI 10 and lysed at 4, 6 and 8 hpi. Lysates were subjected to western blot analysis for PKR, phosphorylated PKR and tubulin. (D) HeLa wt, G3BP1 k.o., G3BP2 k.o. and G3BP1/2 k.o. cells were infected with EMCV-L^{Zn} at MOI 10 and lysed at 4, 6 and 8 hpi. Subsequently RNA was isolated and RT-qPCR analysis was performed for IFN-β, actin mRNA and EMCV vRNA. IFN-β mRNA is depicted as the fold induction of IFN-β mRNA levels compared to mock infected cells, after correction for actin mRNA levels. EMCV vRNA is shown as a copy number per cell, calculated from a plasmid standard. Two-way ANOVA with Bonferroni post-hoc test was used to calculate statistical significance (**** p<0.0001).

The ability of enteroviruses to suppress SG formation was initially attributed to the cleavage of G3BP1 by 3C^{pro} (34–36). In these studies, expression of a cleavage resistant G3BP1 (G3BP1^{Q326E}) led to an increase in the number of SGs (34, 35) and a small reduction in viral titers (34). We also observed that enterovirus 3C^{pro} had an effect on SG formation, but enterovirus 2A^{pro} had a much stronger inhibitory effect on the number of SGs. 2A^{pro} strongly inhibited the number of SGs during overexpression of the protease, and in the context of EMCV infection. While this manuscript was in preparation, another study also reported the essential role of 2A^{pro} in the suppression of SG formation (37). Importantly, in that study it was shown that an EV-A71 mutant containing an IRES between the P1 and P2 region and a catalytically inactive 2A^{pro} was no longer able to suppress SG formation (37). These data, combined with our data, strongly suggest that 2A^{pro}, rather than 3C^{pro}, is the major enterovirus antagonist of the ISR.

How 2A^{pro} suppresses the formation of SGs is unknown. Thus far, no cleavages of known SG components (e.g. G3BP1, G3BP2 and TIA-1) by 2A^{pro} have been detected (35, 37, 38). An alternative mechanism for viruses to prevent SG formation is to interfere with the upstream PKR-dependent signaling (43, 51–55). However, this does not seem to be the case for enteroviruses. It has been reported that PKR and eIF2α are phosphorylated during infection with several enteroviruses (37, 56–58), indicating that the viruses do not block activation of the ISR. Consistently, we did not observe an effect of 2A^{pro} on the phosphorylation of PKR and eIF2α. Furthermore, 2A^{pro} can inhibit arsenite-induced SG formation (Fig 2 and (37)), which is triggered via activation of another eIF2α kinase. This suggests that 2A^{pro} targets SG assembly. While this manuscript was submitted, it was reported that SG formation relies on a newly identified interaction between eIF4GI and G3BP1, and that this interaction is targeted by 2A^{pro} to block SG formation (59). Whether 2A^{pro} also targets other protein-protein interactions underlying SG formation remains to be established. Apart from this, it should be considered that the direct cleavage of eIF4G

by 2A^{pro} contributes to the suppression of SG formation. Cleavage of eIF4G will impair the recruitment of 40S ribosomes to mRNAs, thereby altering the composition of mRNPs which, in turn, may affect their recruitment to SGs. The observation that 2A^{pro} triggers SG formation by cleavage of eIF4G (31, 37, 38) seems counterintuitive, but these 2A^{pro}-induced SGs are smaller in size and differ in composition from traditional SGs. Whether these small SGs fail to “grow” into traditional SGs because of the cleavage of eIF4G by 2A^{pro} or the ability of 2A^{pro} to disrupt protein-protein interactions underlying SG formation, or both, remains unknown.

During enterovirus infection, several signaling molecules in the RLR pathway have been suggested to be cleaved by 2A^{pro} (MDA5, and MAVS) and 3C^{pro} (RIG-I, MAVS, IRF7, and IRF9) (21, 23, 26, 27, 29, 44, 45). There are controversial observations and the question remained which, if any, of the identified cleavages is important for inhibiting IFN- α/β gene transcription. For instance, several studies have shown that 3C^{pro}, when overexpressed, cleaves MAVS and suppresses IFN- α/β induction (24, 25), but we showed that 3C^{pro}, when expressed by a recombinant EMCV, failed to cleave MAVS (21). Here, by infecting cells with EMCV-L^{Zn} encoding 2A^{pro} or 3C^{pro}, we were able to study the effect of these proteases on IFN- α/β mRNA levels in picornavirus-infected cells. Major advantages of this approach are that MDA-5 is activated by a natural ligand (i.e. EMCV dsRNA) and that the enterovirus protease (2A^{pro} or 3C^{pro}) is dynamically expressed during infection, similarly as in enterovirus-infected cells. Using these recombinant viruses, we demonstrate that enterovirus 2A^{pro}, but not 3C^{pro}, suppresses the induction of IFN- α/β gene transcription in HeLa cells. That 3C^{pro}, by cleaving RIG-I, has some role in suppressing IFN- α/β gene transcription in other cell types cannot be excluded. The exact mechanism(s) used by 2A^{pro} to inhibit IFN- α/β gene transcription remains to be identified. We previously demonstrated that 2A^{pro} cleaves MDA5 and MAVS (21). These cleavages are likely involved in the suppression of IFN- α/β induction, but we cannot formally exclude contributions of other 2A^{pro}-dependent mechanisms. For instance, 2A^{pro} is known to affect nuclear-cytoplasmic transport via the cleavage of nuclear pore proteins (NUPs) (60–63), which could interfere with translocation of transcription factors (such as IRF3, IRF7 and NF κ B) to the nucleus. The 2A^{pro}-mediated inhibition of SG formation may also contribute to suppress IFN- α/β induction. In this study, we showed that cells that cannot form SGs (i.e. PKR KD k.o. MEFs and G3BP1/2 k.o. HeLa cells), showed a 5- to 10-fold reduction in IFN- β gene transcription. The observation that heterologous expression of 2A^{pro} during EMCV infection (EMCV-2A^{pro}) nearly completely blocked IFN- β gene transcription (~500-fold reduction) indicates that it is unlikely that suppression of SG formation by 2A^{pro} is the major contributing factor in the viral suppression of IFN- β gene transcription.

Besides 2A proteins as antagonists of antiviral responses, some picornaviruses (like EMCV) also encode an L protein. Many 2A and L proteins have been shown to interfere with antiviral host responses, and have therefore collectively been referred to as “security proteins” (64). Our work demonstrates that for enteroviruses, which lack an L protein, 2A^{pro} is essential for suppressing both SG formation and the induction of IFN- β gene transcription, and we demonstrate that these two antagonizing functions are conserved in 2A^{pro} of different enteroviruses belonging to species EV-A to EV-D. These data support the idea that 2A^{pro} is a major enteroviral “security protein”.

Materials and Methods

Cells and viruses. HeLa R19 and BHK21 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS (V/V). PKR KD and RBD k.o. MEFs have been described previously (46–48). HeLa R19 PKR k.o. have been described elsewhere (43). HeLa R19 G3BP1, G3BP2 and G3BP1/2 k.o. cells were made using CRISPR/cas9 methodology as described previously (43, 65). G3BP1 was targeted with gRNA sequences 5'-TAGTCCCCTGCTGGTCGGG-3' and 5'-TATTACACACTGCTGAACC-3', and G3BP2 was targeted with gRNA sequences 5'-CGCCCTACAAGCAGCGG-3' and 5'-AAGTCCGGAATATTACAC-3'. EV-D68, EV-A71 (BrCr), and CV-A21 (Coe) were obtained from the National Institute for Public Health and Environment (RIVM, The Netherlands). The poliovirus 1 Sabin reference strain was obtained from J. Martin (NIBSC, United Kingdom). All enteroviruses were passaged on HeLa R19 cells and subsequently concentrated by ultracentrifugation through a 30% sucrose cushion at 140,000xg for 16 hours in a SW32Ti rotor and stored at -80°C. Recombinant EMCVs were generated by cloning the genes of interest into the XhoI/NotI restriction sites from the pM16.1-VFETQG-Zn infectious clone that was described previously (43), the Strep2 tag was omitted in the viruses used in this study. Viruses were recovered by transfection of run-off RNA transcripts into BHK-21 cells. Subsequently, viruses were concentrated by ultracentrifugation and stored at -80°C.

Expression plasmids. The 2A^{pro} and 3C^{pro} genes from different enteroviruses were obtained by PCR on enterovirus viral RNA. The oligonucleotides used for these PCR reactions encode flanking XhoI and NotI restriction sites that were used to ligate the PCR products into the desired plasmids. The 3C^{pro} genes were ligated into the pcDNA-GFP vector, while the 2A^{pro} genes were ligated into the pIRES-EGFP-MCS plasmid, both were described previously (42).

Antibodies. The following antibodies were used for IFA staining procedures: α dsRNA (English&Scientific consulting), α G3BP1 (Aviva systems biology), α G3BP2 (Bethyl laboratories), α eIF3 (SantaCruz) and α TIA-1 (SantaCruz). Alexa Fluor 488, Alexa Fluor 594

and Alexa Fluor 647 conjugated secondary antibodies (Invitrogen) were used for detection. For flowcytometry staining we used the α dsRNA (English&Scientific consulting), α p-PKR (Abcam), α p-eIF2 α (Abcam) antibodies and Alexa488 or Alexa647 conjugated (Invitrogen) secondary antibodies. For western blot we used the antibodies: α G3BP1 (Aviva systems biology), α eIF4G (Bethyl laboratories) α MAVS (Alexis-Biochemicals), α RIG-I (Abgent) and α tubulin (Sigma). Respective IRdye680 or IRdye800 conjugated secondary antibodies (LiCOR) were used for detection.

Immune Fluorescence Analysis (IFA). HeLa R19 cells were grown on 12 mm glass coverslips and the next day infected with the indicated viruses (MOI 10) or transfected with 1 μ g of the indicated plasmid using Eugene6 (Promega) according to manufacturer's instructions. At the indicated time points cells were washed in PBS before being fixed in 4% paraformaldehyde for 30 min. Residual paraformaldehyde was washed away using PBS+10 mM Glycine. Cells were permeabilized in PBS+0.1% Triton-X100 and subsequently incubated in blocking buffer (PBS+0.1% Tween-20+3% BSA) for 2 hours, all subsequent steps are performed in blocking buffer. Samples were incubated with primary antibody for 1 hour and incubated with secondary antibodies and DAPI for 30 min. After antibody incubations, samples were washed three times with PBS+0.1% Tween-20. Before mounting coverslips on microscopy slides with FluorSafe (Calbiochem) they were washed once more in milliQ. Cells were examined by confocal microscopy (Leica SPE-II) and Leica Application Suite Advanced Fluorescence software (LAS-AF).

Quantification of SGs. The number of SGs and their surface area in ~100 cells per condition were analyzed by ImageJ software, using a combined total of 10-20 images. For each image, background signal was removed by creating a blurred duplicate and subtracting it from the original image. Subsequently, remaining diffuse (cytoplasmic) SG marker signal was removed via weak blurring, adjustment of the contrast settings and applying a black-and-white threshold. In the resulting image, the number and average surface area of the SGs (shown in black on a white background) were quantified. The used macro is available upon request. Subsequent statistical analyses were performed using GraphPad Prism software. Error bars represent standard deviations, p-values were calculated using one-way ANOVA analysis with Bonferroni post-hoc test (infections with recombinant EMCVs).

Flow cytometry analysis. HeLa R19 cells were seeded in 12-wells plates and the next day infected with the indicated viruses (MOI 10). Six hr p.i. cells were trypsinized and resuspended in FACS buffer (PBS + 1% BSA). Cells were fixed for 30 minutes in 2% paraformaldehyde in FACS buffer and subsequently fixed in ice-cold methanol for 10 minutes. All subsequent steps are performed in FACS buffer. Cells were stained with primary antibodies for 1 hour. Subsequently cells were washed three times and incubated

in secondary antibodies, in the dark, for 30 min. Cells were washed three times and kept in 1% paraformaldehyde until analysis on FACS Canto II (BD biosciences) using BD FACS Diva software. Data analysis was performed using FlowJo software (TreeStar).

Western Blot analysis. HeLa R19 cells were seeded in 10 cm dishes and the next day infected with the indicated viruses (MOI 10). 8 hr p.i. cells were released using trypsin, washed once in PBS and lysed in 100 μ l lysisbuffer (100 mM Tris pH 8.0, 1 mM EDTA, 50 mM NaCl, 1% NP40, protease inhibitor mix (Roche)). Post nuclear lysate was obtained by centrifugation at 15000xg at 4 °C for 15 min. The amount of total protein in the lysates was determined using BCA assay (ThermoFisher). 50 μ g protein from the cleared cell lysates were resolved using reducing sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.2 μ m nitrocellulose membranes by wet electrophoretic transfer. Membranes were incubated 1h in blocking buffer (PBS + 0.1% Tween 20 + 2% BSA) and successively incubated overnight with primary antibodies diluted in blocking buffer and then for 30 min with respective secondary antibodies diluted in blocking buffer. After antibody incubations, the membranes were washed three times with PBS+0.1% Tween-20. Finally, membranes were washed once with PBS and scanned using an Odyssey Imager (Li-COR).

RT-PCR analysis. HeLa R19 cells were seeded in 24-wells plates and the next day infected with the indicated viruses at MOI 10, 8 hr p.i. cells were lysed and cellular RNA was isolated using total RNA isolation kit (Machery-Nagel) according to manufacturer's instructions. Reverse transcription was set up using TaqMan Reverse Transcription Reagents (Applied Biosystems) before performing qPCR analysis with SYBR green (Roche) as described previously (43).

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Chapter 6

The Aichivirus leader protein inhibits the integrated stress response by preventing p-eIF2-mediated inhibition of eIF2B function

6

Linda J. Visser^{1,*}, Huib H. Rabouw^{1,*}, José G. Dekker¹, Piero Giansanti^{2,*}, Jesús G. Saucedo¹, Susanne G. van der Grein¹, Martijn A. Langereis¹, Albert J.R. Heck², Raoul J. de Groot¹, Frank J.M. van Kuppeveld^{1,#}

¹Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

²Biomolecular Mass Spectrometry and Proteomics, Utrecht Institute for Pharmaceutical Sciences and Bijvoet Centre for Biomolecular Research, Utrecht University, Utrecht, The Netherlands

*Present Address: Chair of Proteomics and Bioanalytics, Technical University of Munich, Freising, Germany

*These authors share first authorship.

#Corresponding author.

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Abstract

Under conditions of stress, eukaryotic cells activate the integrated stress response (ISR) to temporarily block translation initiation, and restore cellular homeostasis. The ISR can be activated by four stress kinases, which, upon sensing specific stress stimuli, converge on phosphorylation of translation initiation factor eIF2. p-eIF2 sequesters the eIF2-specific guanine exchange factor (GEF) eIF2B, which prevents eIF2•GDP/GTP exchange, an essential step in translation initiation. Many viruses avoid the detrimental effects of the ISR on viral protein synthesis by encoding specific ISR antagonists. We describe the mechanism through which aichivirus (AiV; family picornavirus, genus *Kobuvirus* KoV), interferes with the ISR. Whereas almost all ISR antagonist prevent or reverse eIF2 phosphorylation, AiV allows ongoing translation in the presence of high concentrations of p-eIF2, through the function of its leader protein (AiVL). AiVL binds eIF2B and blocks subsequent association of p-eIF2, thus preventing p-eIF2 from interfering with eIF2B GEF function. Besides AiVL, also the closely related mouse KoV leader, but not other KoV leaders, inhibits the ISR, suggestive of a relatively recent gain-of-function event. A mode of action similar to that of AiVL has been described for a phylogenetically unrelated coronavirus accessory protein (AcP10), which shares no similarity with AiVL. Thus, the ability to bind eIF2B and render it insensitive to p-eIF2 was acquired by at least two unrelated virus species through convergent evolution.

Introduction

To ensure efficient virus propagation, viruses evade several antiviral pathways within the infected host cell. One of these pathways, the integrated stress response (ISR), can be activated in response to a variety of intracellular danger signals including viral infection. The ISR mediates a temporal stop in translation, conserving energy and redirecting cellular resources towards resolving the stress situation (1). In mammalian cells, four ISR-sensor proteins have been described. Protein kinase R (PKR) detects cytoplasmic dsRNA, and is considered the most important ISR sensor protein during virus infections (2). PKR-like ER-resident kinase (PERK) responds to ER-stress that causes misfolded proteins to accumulate in the ER lumen (3, 4). General control non-derepressible (GCN2) is best known for its ability to detect nutrient deprivation and subsequent accumulation of uncharged tRNA molecules (5). Finally, Heme-responsive inhibitor (HRI) is activated by heme deficiency or the presence of toxic heavy metals (6, 7). Upon sensing their respective activating trigger, these proteins homodimerize and autophosphorylate, and subsequently phosphorylate translation initiation factor eIF2. Phosphorylated eIF2 (p-eIF2) tightly binds the eIF2-specific guanine exchange factor (GEF) eIF2B, thereby inhibiting eIF2 recycling and preventing translation initiation. The stalled translation initiation complexes then condense to form cytoplasmic foci called stress granules (SGs).

In virus infected cells, ISR-mediated inhibition of translation severely represses production of viral proteins, and consequently the generation of progeny virus. Therefore, viruses have evolved multiple ways to circumvent the ISR, either by preventing eIF2 α kinase activation (Class 1 antagonist), by preventing eIF2 α phosphorylation (Class 2 antagonist), or by actively inducing dephosphorylation of p-eIF2 (Class 3 antagonist) (8). Recently, a novel mode of action was described for a coronavirus accessory protein, AcP10, that rescues cellular and viral translation without affecting eIF2 α phosphorylation by preventing the formation of p-eIF2•eIF2B complexes (Class 4 antagonist, (8)).

The picornavirus family encompasses a large group of non-enveloped positive-strand RNA viruses. This family includes many well-known pathogens of relevance to the health of humans and animals, such as enteroviruses (e.g. poliovirus), hepatoviruses (e.g. hepatitis A virus), and aphthoviruses (e.g. foot-and-mouth disease virus), and cardiociruses (e.g. encephalomyocarditis virus). Picornaviral genomes are 7,5-8 kb in length, and contain a single open reading frame that encodes a large polyprotein. This polyprotein is processed by viral proteases to yield the structural capsid proteins (VP1-4) and various proteins involved in replication (2A-2C and 3A-3D). Besides this conserved set of proteins, several picornaviruses also encode a leader (L) protein upstream of the capsid coding region. L proteins are highly diverse in sequence, structure, and function, and are considered to be

accessory proteins involved in optimizing virus replication, e.g. through suppression of the host cell's antiviral responses (9). The best known are the L protease of FMDV, which cleaves eIF4G to mediate host shut-off (10), and the L proteins of cardioviruses, which block IFN induction and stress responses (11, 12).

In 1991, a novel species of picornavirus, called aichivirus (AiV), was described as the causative agent of outbreaks of gastroenteritis in humans (13). Aichivirus was classified within a novel genus of picornaviruses, *Kobuvirus* (KoV). Since then, related KoVs have been found in various other mammalian species. KoVs are further subdivided into the lineages Aichi A (e.g. Aichivirus), Aichi B (e.g. Bovine KoV), and Aichi C (e.g. Porcine KoV) viruses, based on sequence analyses of their 3CD-polymerase genes. In this study, we sought to determine whether and how KoVs evade inhibition by the ISR. We show that AiV actively blocks the ISR, both when activated in a PKR-dependent or HRI-dependent manner. Overexpression experiments demonstrate that the AiV-encoded L protein (AiVL) is a highly potent ISR antagonist in the absence of other viral proteins. Consistently, a recombinant AiV lacking a functional AiVL protein induces full-blown ISR activity, indicating that AiVL is the main, and possibly the sole ISR antagonist encoded by AiV. AiVL does not counteract the upstream signaling events of the ISR pathway, but rescues global translation in the presence of (high levels of) p-eIF2. This effect is mediated by its association to eIF2B, which prevents subsequent binding of p-eIF2, and thus renders eIF2B insensitive to inhibition by p-eIF2. We show that the closely related mouse kobuvirus L protein (M-KoVL) has a similar function, but that other tested KoVLs do not, suggesting that the ISR antagonist function was obtained through a gain-of-function event relatively late in the KoVL evolutionary trajectory. AiVL's mode of action strongly resembles that of Beluga-whale coronavirus AcP10, and defines it as a Class 4 ISR antagonist (8). Thus, the ability to bind eIF2B and prevent inhibition by p-eIF2 independently developed within at least two unrelated viruses, in non-homologous proteins, through convergent evolution.

Results

Aichivirus prevents ISR activity in infected cells. To test whether AiV suppresses the ISR in infected cells, we assessed SG formation in AiV-infected cells. For this, we first established replication kinetics in infected Vero cells. A single-step growth curve, in which we quantified the production of progeny infectious virus at the indicated time points pi. (Fig 1A), indicated that most virus is produced between 6 and 10 h pi. In line with these data, an immunofluorescence assay (IFA) shows large quantities of dsRNA, indicative of active virus replication, at the same time points (Fig 1B). Interestingly, despite the appearance of dsRNA, which could activate the ISR sensor protein PKR, infected cells were consistently

devoid of SGs throughout the infection cycle, up to and including the moment they started displaying cytopathic effects at 10 h pi. These data provided first indication that AiV counteracts the ISR. This was further corroborated by the observation that AiV-infected cells failed to assemble SGs in response to arsenite treatment, which activates the eIF2 α kinase HRI (Fig 1C).

The Aichivirus-encoded L protein rescues cellular translation in the presence of p-eIF2.

Multiple picornavirus L proteins have been shown to repress cellular antiviral responses, including the ISR (11, 12, 14, 15). To test whether the AiV Leader protein (AiVL) is responsible for inhibiting the ISR in AiV-infected cells, we transiently overexpressed EGFP-tagged AiVL in absence of other viral proteins, and assessed SG formation in response to (i) the pEGFP plasmid DNA transfection itself, which induces PKR- and p-eIF2-dependent stress in some of the transfected cells (Fig 2A,(16, 17)), (ii) treatment with sodium arsenite, which induces HRI- and p-eIF2-dependent stress in all treated cells (Fig 2B), or (iii) treatment with pateamine A, which blocks translation independently of p-eIF2 (Fig 2C). Expression of AiVL prevented p-eIF2-mediated SG formation (Fig 2A and 2B), but not p-eIF2-independent SG formation induced by pateamine A (Fig 2C), indicating that AiVL is indeed a functional ISR antagonist. Furthermore, these data suggest that AiVL targets a downstream step in the ISR pathway that is shared by transfection-induced and arsenite-induced ISR activity. To determine which step of the ISR is targeted by AiVL, we performed flow cytometry analyses to quantify p-eIF2 α levels and active translation rates in cells overexpressing AiVL. pEGFP plasmid DNA transfection induces elevated levels of p-eIF2 in a portion of the transfected cells, while the remainder of the cells are unstressed (16, 17), resulting in a mixed population of transfected cells with either high (shown in red) or low (shown in green) levels of p-eIF2 α (Fig. 2D). The level of p-eIF2 α was similar between AiVL expressing cells and cells expressing negative control EGFP, suggesting that AiVL neither impacts eIF2 α phosphorylation, nor induces eIF2 α dephosphorylation. As expected, EGFP expressing cells with high p-eIF2 α levels displayed impaired translation rates (as measured by puromycin labeling) (Fig 2D, lower panels). In contrast, in AiVL expressing cells p-eIF2 α had no detectable effect on translation, suggesting that AiVL renders cellular translation insensitive to eIF2 α phosphorylation.

A putative Zn-finger domain is essential for ISR antagonism by AiVL. To obtain a non-functional AiVL mutant, we performed alanine screening, and substituted specific tryptophans, histidines, cysteines, or potential phosphorylation sites by alanines (Fig 3A, residues indicated in red). The mutant proteins were overexpressed as EGFP fusion proteins and tested for their ability to inhibit arsenite-induced SG formation using IFA. Out of the twenty mutations tested, five (H20A, H24A, H40A, C53A, and C56A) rendered the AiVL non-functional, three of which (H24A, C53A, C56A) did so without significantly

impacting AiVL-expression level or its intracellular localization (Fig 3B). A full overview of the mutants tested is given in supplementary figure S1. Flow cytometry analysis confirmed that the mutant AiVL proteins failed to rescue translation in the presence of elevated p-eIF2 α levels (Fig 3C). Interestingly, two of the inactivating mutations affected residues that make up an *in silico* predicted Zn-finger ribbon (C53 and C56). Most Zn-fingers consist of two C/H - X₂₋₄ - C/H motifs, which mediate a quadruple interaction with a divalent cation such as Zn²⁺. In the case of AiVL, the second C/H - X₂₋₄ - C/H motif was not predicted *in silico*, but may consist of H20 and H24, since both these residues were also found to be essential for AiVL's function as ISR antagonist (Fig 3B and S1).

Figure 1: Aichivirus counteracts the ISR. Vero cells were infected with aichivirus at a multiplicity of infection (MOI) of 2. **(A)** At the indicated time points, progeny infectious virus particles were harvested and quantified by end-point titrations. **(B)** At the indicated time-points, cells were fixed and stress granule (SG) formation was analyzed by immunofluorescence using an antibody directed against SG marker G3BP1. dsRNA staining was used to identify cells that were actively replicating AiV. **(C)** Aichivirus-infected cells were treated with 1 mM arsenite from 7 hrs pi. until 8 hrs pi., and SG formation was analyzed as in **(B)**.

Figure 2: AiVL rescues translation in the presence of p-eIF2. HeLa cells were transfected with plasmids encoding EGFP or AiVL-EGFP. Subsequently, SG formation was monitored by immunofluorescence in cells without additional treatment **(A)**, cells treated with 250 μ M arsenite for 1 hour **(B)**, or cells treated with 100 nM pateamine A for 1 hour **(C)**. **(D)** Cells were transfected with pEGFP or pEGFP-AiVL. Next day, active translation was labeled using 20 μ g/ml puromycin for 15 min. Subsequently, cells were fixed and analyzed by flow cytometry for p-eIF2 α content and the level of puromycin incorporation. Shown are cells positive for EGFP, with either low p-eIF2 α levels (indicated in green) or high p-eIF2 α levels (indicated in red).

Figure 3: A putative Zn-finger domain is essential for ISR antagonism by AiVL. (A) Schematic representation of the AiV genome. The position of the gene encoding AiVL is indicated in green. The amino acid sequence of AiVL is shown in the bottom panel, and each residue that was mutated in the alanine screening approach is indicated in red. (B) The AiVL mutants were overexpressed in cells, and their ability to prevent SG formation upon arsenite treatment was analyzed by immunofluorescence using antibodies directed against G3BP1. A small selection out of the total panel of mutants is shown. See Fig S1 for a complete overview. (C) wt AiVL, as well as AiVL mutants H24A, C53A, and C56A, were transiently overexpressed, and after overnight incubation, active translation was labeled using 20 µg/ml puromycin for 15 min. Cells were subsequently analyzed by flow cytometry for active translation rates as described in Fig 2D.

Figure 4: AiVL is the main antagonist encoded by aichivirus. (A) Vero cells were infected with the indicated recombinant aichiviruses at an MOI of 10 for 8 hrs. Translation was labeled with 20 µg/ml puromycin during the final 15 min of the experiment. Puromycin incorporation, and p-eIF2α content were measured by flow cytometry. (B) Vero cells were infected with the indicated recombinant aichiviruses at an MOI of 1 for 8 hrs. Cells were either left untreated (top panels), or treated with 1.5 mM arsenite during the final hour of the experiment. dsRNA content and the presence of SGs were assessed by IFA using antibodies directed against dsRNA and G3BP1, respectively.

AiVL is the main antagonist encoded by Aichivirus. To corroborate our findings that AiVL functions as ISR antagonist rescuing translation in the presence of p-eIF2 α , and to show that it functions in the context of AiV infection, we created a recombinant AiV in which the L protein was inactivated by two point mutations (C53A & C56A). Vero cells were infected with wt AiV or AiV-L-C53A/C56A, and analyzed by flow cytometry for p-eIF2 α levels and active translation rates (Fig 4A), or by IFA for dsRNA content and SG formation (Fig 4B), at 8 hrs pi. Cells infected with AiV or AiV-L-C53A/C56A contained comparable quantities of dsRNA and similar levels of p-eIF2 α , indicating efficient virus replication and ISR activation. However, cells infected with AiV-L-C53A/C56A –but not with wt virus– displayed impaired translation rates and contained SGs. These data unambiguously show that AiVL is an ISR antagonist that rescues translation in the presence of elevated p-eIF2 α levels in AiV-infected cells.

AiVL interacts with translation initiation factor eIF2B. To determine how AiVL rescues translation, we identified its cellular interaction partners by mass spectrometry analysis. HEK293T cells were transiently transfected with plasmids encoding tandem affinity purification (TAP) tagged AiVL. AiVL and its cellular interaction partners, were purified as described previously (8) and analyzed by LC-MS/MS. Two out of five eIF2B subunits (eIF2B γ and eIF2B δ) were modestly but significantly enriched in AiVL pull-down samples relative to empty vector control samples (Fig 5A). To confirm the interaction with eIF2B, AiVL purification samples were analyzed by Western blot using an antibody directed against eIF2B α . Indeed, eIF2B α co-purified with AiVL, albeit to a much lesser extent than when using Beluga whale-CoV AcP10, which was included as positive control (Fig 5B). Importantly, no interaction was found between the phenotypically inactive mutant AiVL C56A and eIF2B.

> **Figure 5: AiVL interacts with translation initiation factor eIF2B.** (A) Volcano plot showing the proteins differentially co-immunoprecipitated with TAP-AiVL pull-down compared to empty vector control. Significantly enriched proteins as determined by a t-test (5% permutation-based FDR) are indicated in shades of red that indicate the values set for so. SAINT scores for each of the enriched proteins are indicated by the blue colored labels. (B) The indicated TAP-tagged proteins were transiently overexpressed in HEK293T cells. These proteins were subsequently purified as described previously (8). The final wash step was performed either in low-salt buffer to keep the eIF2-eIF2B interaction intact (left panels), or in high salt buffer (500 mM KCl) to disrupt eIF2-eIF2B interactions (right panels). For each sample, the bead-associated protein fraction (indicated as “B”), and the supernatant fraction (indicated as “S”) is shown. The presence of co-purified eIF2 and eIF2B was analyzed by Western blotting, using antibodies directed against their respective α -subunits. (C) The indicated EGFP-tagged proteins were transiently overexpressed in HeLa-R19-eIF2B β -3Xflag cells. Subsequently, eIF2B complexes were purified from cell lysates as described previously (8). The washes were performed the same way as in (B), and again the bead-associated protein fraction (indicated as “B”), and the supernatant fraction (indicated as “S”) are shown for each sample. The presence of co-purified eIF2 (represented by its α -subunit) and AcP10/ AiVL (assessed using an antibody directed against EGFP) was analyzed by Western blotting.

As reported, purification of AcP10 also indirectly co-purifies eIF2 α , since the eIF2•eIF2B macrocomplex remains intact during this purification procedure (8, 18). No eIF2 α band was observed in the AiVL pulldown sample, but given the low co-IP efficiency when using AiVL as bait, it is to be expected that the eIF2 α signal may fall below the detection limit even if eIF2 α is in fact present (Fig 5B). To address the possibility that AiVL may interact with eIF2 rather than eIF2B, we disrupted the eIF2•eIF2B interaction using a high-salt wash (500 μ M KCl) (Fig 5B, right panels), and found that eIF2B still associates with AiVL under these conditions. This demonstrates that AiVL directly binds eIF2B, independent of the presence of eIF2.

As a complimentary approach, we pulled down eIF2B from HeLa cells stably expressing flag-tagged eIF2B β (HeLa-eIF2B β -3Xflag, (19)), and analyzed co-purified eIF2 α and AiVL by Western blot (Fig 5C). AiVL, like AcP10, could be detected in eIF2B β pulldown samples, again suggesting that AiVL interacts with eIF2B. A high-salt wash step that disturbs the eIF2•eIF2B interaction, released most of the eIF2 into the supernatant fraction, but had only a minor impact on the association of AiVL (Fig 5C, compare lanes 1 and 2 with lanes 7 and 8). Taken together, the data suggest that AiVL rescues translation in presence of p-eIF2 through its binding to eIF2B. The amount of eIF2B α co-purified with AiVL is consistently low (Fig 5B), while the reverse IP using eIF2B as bait efficiently yields AiVL (Fig 5C). This is likely caused by a vast excess of AiVL protein over eIF2B complexes upon transient overexpression, which causes most AiVL to be free of eIF2B. Additionally, and unlike the AcP10•eIF2B interaction, the AiVL•eIF2B association may be unstable, and less likely to survive the lengthy TAP-AiVL pulldown procedure (~20 hrs) than the shorter eIF2B-flag pulldown procedure (~3 hrs). A relatively unstable AiVL•eIF2B interaction can also be deduced from the observation that incubation of AiVL•eIF2B complexes in wash buffer for 15 min released approximately half of the AiVL protein from eIF2B into the supernatant fraction (Fig 5C, lanes 1 and 2).

AiVL counteracts the association of p-eIF2 to eIF2B. AcP10 binding to eIF2B prohibits the inhibition of eIF2B GEF function through competitive displacement of p-eIF2 (8). To test the effects of AiVL on the formation of p-eIF2•eIF2B complexes, we transfected HeLa-eIF2B β -3Xflag cells with plasmids encoding AiVL or control proteins, and treated them with arsenite to induce eIF2 phosphorylation. We subsequently pulled down eIF2B from cell lysates and analyzed the amount of co-immunoprecipitated p-eIF2 α by Western blotting. AcP10 expression interfered with p-eIF2•eIF2B complex formation, resulting in a significant reduction in the amount of p-eIF2 α bound to eIF2B ((8), Fig 6A). AiVL expression has a similar effect, suggesting that AiVL –like AcP10– keeps eIF2B active despite eIF2 phosphorylation by counteracting p-eIF2•eIF2B complex formation. The remaining p-eIF2 α signal (approximately 27% of the control sample) corresponds to the background

Figure 6: AiVL counteracts the association of p-eIF2 to eIF2B. The indicated EGFP-tagged proteins were transiently overexpressed in HeLa-R19-eIF2B β -3Xflag cells. Cells were treated with 250 μ M arsenite for 1 hr to induce phosphorylation of eIF2 α . (A) eIF2B complexes were purified from cell lysates as described previously (8). The presence of co-purified eIF2, p-eIF2, and AcP10/AiVL was assessed by Western blotting using antibodies directed against eIF2 α , p-eIF2 α , and EGFP, respectively. (B) Flow cytometry analysis of the transfection efficiencies obtained in the experiment shown in (A). (C) HEK293T cells were transiently transfected with plasmids encoding the indicated TAP-tagged proteins. Cells were subsequently treated with the indicated arsenite concentrations for 1 hr, and the overexpressed proteins were purified from cell lysates. The presence of co-purified eIF2, p-eIF2, and eIF2B was assessed by Western blotting using antibodies directed against eIF2 α , p-eIF2 α , and eIF2B α , respectively.

level that is expected at the transfection efficiencies of ~70% obtained in this experiment (Fig 6B). Our data thus suggest that the AiVL•eIF2B interaction prevents p-eIF2 binding to eIF2B. This effect is p-eIF2-specific, since the total amount of eIF2 bound to eIF2B was unaffected by the presence of AiVL.

Next, we overexpressed TAP-tagged AiVL or control proteins in HEK293T cells that were subsequently treated with several arsenite concentrations to induce increasing levels of p-eIF2. We then tandem affinity purified the overexpressed proteins and assessed co-purified eIF2B and (p-)eIF2, using antibodies directed against eIF2B α , eIF2 α and p-eIF2 α (Fig 6C). In line with previous data (8), the efficiency of eIF2B co-purification with AcP10 is lower when cells were treated with arsenite to induce eIF2 phosphorylation, indicative of a competition between AcP10 and p-eIF2 for eIF2B binding. In contrast, AiVL•eIF2B complexes, while present in relatively low quantities, were not affected by p-eIF2 concentrations. Importantly, neither AcP10•eIF2B, nor AiVL•eIF2B complexes contained detectable amounts of p-eIF2, again indicating that AiVL counteracts the p-eIF2•eIF2B interaction. Collectively, our data indicate that AiVL has a similar effect as AcP10, rendering eIF2B GEF function insensitive to inhibition by p-eIF2, but some aspects of AiVL's interaction with eIF2B are remarkably different from those of AcP10. First, the amount of eIF2B co-purified with AiVL is consistently and significantly lower than with AcP10 (Fig 5B and 7). Second, AiVL's interaction with eIF2B seems relatively unstable (Fig 5C, lanes 1 and 2). Third, the AiVL•eIF2B interaction is insensitive to high levels of p-eIF2 (Fig 6). Fourth, AiVL is a highly soluble protein, in stark contrast to AcP10, which was shown to largely aggregate in insoluble perinuclear deposits upon overexpression (8).

> **Figure 7: Mouse KoVL, but not the KoVLs from other species, also acts as ISR antagonist.** (A) phylogenetic tree of kobuvirus L proteins. The scale bar represents the number of substitutions per site. (B) Analysis of the ISR antagonist function of the indicated kobuvirus L proteins in HeLa cells by IFA. Shown is the percentage of cells that contain SGs after treatment with 250 μ M arsenite for 1 hr, within the population of transfected cells (EGFP positive, shown in green), as well as untransfected cells from the same wells (EGFP negative, shown in grey). (C) HeLa cells were transiently transfected with plasmids encoding the indicated EGFP tagged proteins and subsequently analyzed by flowcytometry as in Fig 2D and 3C. Plotted is the ratio in mean fluorescence intensity (MFI) values of the puromycin staining, between the p-eIF2 α ^{high} (stressed) and p-eIF2 α ^{low} (unstressed) cell populations. A ratio of 1 (indicated by the dotted line) indicates that the presence of p-eIF2 α has no impact on translation, and thus that the overexpressed protein renders translation insensitive to p-eIF2. (D) HeLa cells transiently overexpressing the indicated EGFP tagged proteins were treated with 250 μ M arsenite for 1 hr, and translation was labeled using puromycin during the final 15 min of the experiment. Puromycin incorporation was analyzed by flowcytometry. Plotted is the ratio in mean fluorescence intensity (MFI) values between the EGFP^{high} (transfected) and EGFP^{low} (untransfected) cell populations. A ratio of 1 (indicated by the dotted line) indicates that the overexpressed protein has no impact on translation in stressed cells. Values higher than 1 indicate that the overexpressed protein rescues translation in arsenite-treated cells. (E) The indicated TAP-tagged proteins were overexpressed in HEK293T cells and purified as described above. The presence of co-purified eIF2B was analyzed by Western blotting using antibodies directed against eIF2B α . (F&G) LR7 cells or PD-5 cells were transfected with plasmids encoding the indicated EGFP-tagged proteins. SG formation in response to treatment with 500 μ M arsenite for 1 hr was assessed by IFA and plotted as in (B).

Mouse KoVL, but not the KoVLs from more distantly related species, can also antagonize the ISR. Besides AiVL, the kobuvirus family consists of viruses that infect mammalian species such as mice (Mouse KoV), dogs (Canine KoV), pigs, (Porcine KoV), and cattle (Bovine KoV). Each of these viruses contains an L protein homologous to AiVL (Fig 7A), with amino acid identities ranging from ~55% to ~80%. To test whether the ISR inhibiting function of AiVL is conserved for L proteins throughout the *Kobuvirus* genus, we separately overexpressed the L proteins of AiV, Mouse KoV (M-KoVL), Canine KoV (C-KoVL), Roller KoV (R-KoVL), Bovine KoV (B-KoVL), Ferret KoV (F-KoVL), and Porcine KoV (P-KoVL) in HeLa cells and tested their ability to counteract arsenite-induced SG formation.

Surprisingly, only Mouse KoV-L –which is phylogenetically most related to AiVL– was able to prevent SG formation (Fig 7B). These data were corroborated by FACS experiments showing that AiVL or M-KoVL expressing cells display unimpaired translation rates irrespective of the phosphorylation status of eIF2, either upon DNA transfection alone (Fig 7C), or after arsenite treatment (Fig 7D). Furthermore, in a KoVL co-IP experiment we show that only AiVL and M-KoVL detectably interact with eIF2B (Fig 7E). Consistently, the four residues that may form the Zn-finger structure (H20, H24, C53, and C56) and are essential for AiVL's ISR antagonist function (Figs 3 and S1), are strictly conserved in AiVL and M-KoVL, whereas the other KoVLs lack residues homologous to H20 and H24 in AiVL. Despite high conservation of the eIF2B subunits between mammalian species, we could not exclude the possibility that the KoVL proteins interact specifically with eIF2B from their respective host species. Therefore, we tested ISR antagonism by a selection of KoVL proteins (AiVL, M-KoVL, C-KoVL, and P-KoVL) in cell lines of mouse (LR7) and pig (PD-5) origin, using transient overexpression and subsequent analysis of arsenite-induced SG formation. In both LR7 cells (Fig 7F) and PD-5 cells (Fig 7G), only AiVL and M-KoVL are functional ISR antagonists, demonstrating that host species specificities do not underlie the differences in ISR antagonism between KoVL proteins. Rather, these data suggest that besides AiVL and M-KoVL none of the KoVLs function as ISR antagonist. Thus, the acquisition of AiVL's ISR antagonist function was likely a relatively recent evolutionary event that occurred within the Aichi A lineage.

Discussion

We set out to investigate how kobuviruses, and AiV in particular, counteract antiviral ISR activity. We determine that AiV encodes a highly potent ISR antagonist (AiVL) that prevents cellular translational arrest in the presence of p-eIF2. AiVL binds translation initiation factor eIF2B, and thereby specifically prevents subsequent association of p-eIF2. Consequently, in the presence of AiVL, eIF2B preferentially binds its substrate (non-phosphorylated eIF2) over its inhibitor (phosphorylated eIF2), and thus remains active even under conditions of

stress. This mode of action is similar to that of the recently described beluga whale-CoV protein AcP10, and differs from that of all other known ISR antagonists. AcP10 and AiVL are prototypes of a new Class of ISR antagonist, that targets the ISR downstream of eIF2 phosphorylation, at the level of eIF2B GEF function inhibition. ISR inhibition by precluding the p-eIF2•eIF2B interaction thus appeared at least twice, in phylogenetically distinct virus families, through convergent evolution.

AiVL and AcP10 both bind eIF2B, and correspondingly, they localize to the cytoplasm. However, AiVL has a diffuse localization, whereas AcP10 is largely aggregated in insoluble perinuclear deposits. Consequently, only low AcP10 concentrations remain soluble and can participate in competition with p-eIF2 for eIF2B binding (8), while soluble AiVL levels may vastly exceed those of p-eIF2 or eIF2B (Fig S2). Furthermore, in contrast to AcP10, AiVL has a low stability interaction with eIF2B, purification of AiVL yields little co-purified eIF2B, and AiVL was not detectably displaced from eIF2B by high p-eIF2 levels. These differences between AiVL and AcP10 may be related to their difference in solubility. First, since soluble AiVL is present in vast excess over eIF2B, most AiVL will not be bound to eIF2B and thus eIF2B co-IP is expected to be inefficient when using AiVL as bait (Figs 5 and 6). AcP10 on the other hand is largely insoluble and thus the levels of soluble AcP10 available for eIF2B binding remain relatively low. Second, increasing p-eIF2 concentrations might be unable to outcompete AiVL due to a large excess of soluble AiVL over p-eIF2, even under severe stress conditions (Fig 6). Third, given such high levels of AiVL, a relatively low stability eIF2B interaction would suffice to prevent inhibition of eIF2B activity (Fig 5).

ISR antagonists can be classified in several categories based on which signal transduction event in the ISR pathway they counteract. Most ISR antagonists are specific to a particular eIF2 α kinase, preventing either its activation (class 1 antagonist) or its kinase activity on eIF2 α (class 2 antagonist). However, virus infection often causes extensive changes in the intracellular environment and may activate more than one eIF2 kinase. Thus, these antagonists may fail to prevent ISR activity entirely. Alternatively, an antagonist may induce dephosphorylation of p-eIF2 (class 3 antagonist), which counters the ISR irrespective of the kinase involved. However, this approach only mediates a reduction in p-eIF2 levels to some extent based on the cell's capacity for eIF2 dephosphorylation, and thus the ISR may still signal under conditions of severe stress (20, 21). AiVL does not belong to any of these classes, and instead renders eIF2B immune to eIF2 phosphorylation. Thereby, it blocks the ISR regardless of the nature or severity of the stress trigger, or the eIF2 kinase(s) involved. Thus, AiVLs mode of action is arguably the most effective way to block the ISR described so far.

RNA viruses typically obtain their genes via recombination events, and thus the chance of acquiring antagonists with a certain mode-of action depends on the availability of other

(cellular) RNAs that encode proteins with such functions. For example, dsRNA binding domains are relatively common, so recombination events that lead to the acquisition of dsRNA binding proteins should occur often. In line with this, many viruses encode specific PKR inhibitors that act by sequestering dsRNA (17, 22–25). In contrast, no host cell proteins have been described that bind eIF2B and interfere with p-eIF2 binding, and thus a protein with this mode of action is unlikely to be obtained by viruses. In line with this, both viral proteins that prevent p-eIF2 from inhibiting eIF2B, AcP10 and AiVL, seem to have obtained their ISR antagonist function via gain-of-function substitutions in pre-existing viral proteins, rather than via recombination with RNAs encoding eIF2B-binding proteins. Our data indicate that most L proteins encoded by related viruses in the KoV family do not function as ISR antagonists. Thus, it is highly likely that the ancestral KoVL protein was not an ISR inhibitor, and that this function was obtained relatively recently within the AiV A lineage. Remarkably, since we also demonstrate that Aichivirus has no ISR antagonists besides AiVL, the other KoVs must either lack ISR antagonists altogether, or encode an antagonist(s) that is absent in AiV.

The ISR plays a role in a variety of different cell biological processes, both during normal cell function and under conditions of stress (26–29). Its activity is typically balanced and proportional to the situation at hand, and deviations from this balance, both towards a hypoactive or hyperactive ISR, may have pathological consequences (30–32). Most notoriously, a variety of neurodegenerative diseases have been linked to an overactive ISR, including but not limited to Parkinson's disease, Alzheimer's disease, Vanishing White Matter, and prion disease (33–37). A causal connection was even established between neuropathology and an overactive ISR by showing that ISR inhibitors may alleviate neurotoxicity (28, 33, 36, 38–40). Viruses – through their rapid rates of evolution over extensive periods of time, and under strong selective pressure to countermeasure the ISR – could teach us the most effective ways to interfere with ISR signaling. The identification of novel types of ISR antagonists like AiVL and AcP10 and elucidation of the details of their mode of action, may therefore provide the basis for novel strategies to modulate the ISR for therapeutic purposes.

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Materials & Methods

Chemical compounds. ISRIB (SML0843) and puromycin (P9620) were obtained from Sigma-Aldrich and used at a final concentration of 200 nM and 20 μ g/mL, respectively. Sodium arsenite was purchased at Riedel-de-Haën (UN1686).

Cells and Viruses. HEK293T, HeLa-R19, and Vero cells were maintained in DMEM (Lonza) supplemented with 10% FCS and penicillin–streptomycin (100 units/mL and 100 μ g/mL). To create recombinant AiV-C⁵³A/C⁵⁶A, the double mutation was introduced in the AiV infectious clone pAV (41) by site-directed mutagenesis. The infectious clones were linearized by HindIII digestion and infectious RNA was synthesized by a T7 *in vitro* transcription reaction (Promega). Infectious RNA was transfected in Vero cells using Lipofectamine 2000 reagents (Invitrogen), and AiV stocks (encoding either wt AiVL or AiVL C⁵³A/C⁵⁶A) were harvested three days post transfection. Integrity of the AiVL gene was confirmed by sanger sequencing, and virus titers were determined using end-point dilution assays on Vero cells.

Immunofluorescence Assay. Immunofluorescence assays were performed as described previously (17), using primary antibodies rabbit- α -G3BP2 (A302-040A, 1:200; Bethyl Laboratories) mouse- α -dsRNA J2 (1:1000; English Scientific & Consulting), rabbit- α -G3BP1 (ARP37713-T100, 1:200; Aviva), goat- α -eIF3 η (sc-16377, 1:200; SantaCruz) and secondary antibodies donkey- α -mouse-Alexa488 (A-21202, 1:200; Thermo Fisher Scientific), donkey- α -goat-Alexa568 (A-11058, 1:200; Thermo Fisher Scientific), donkey- α -rabbit-Alexa647 (A-31573, 1:200; Thermo Fisher Scientific).

Western Blot Analysis. Western blot assays were performed as described previously (17), using primary antibodies rabbit- α -eIF2 α (9722, 1:1000; Cell Signaling), rabbit- α -p-eIF2 α (ab32157, 1:1000; Abcam), mouse- α -tubulin (T9026, 1:2000; Sigma-Aldrich), rabbit- α -eIF2B α (18010-1-AP, 1:1000; Protein Tech), mouse- α -Flag (F3165, 1:1000; Sigma-Aldrich), or rabbit- α -EGFP (42), and secondary antibodies goat- α -mouse-IRDye680 (926-68070, 1:15,000; LI-COR) or goat- α -rabbit-IRDye800 (926-32211, 1:15,000; LI-COR).

Flow Cytometry Analysis of eIF2 α Phosphorylation. Cells were released using trypsin and fixed with paraformaldehyde (4% in PBS) for 20 min. Cells were washed with FACS buffer (PBS + 1% BSA) and subsequently incubated for 10 min in ice-cold methanol. Cells were washed once with FACS buffer and incubated with primary antibodies rabbit- α -p-eIF2 α (ab32157, 1:100; Abcam) and mouse- α -puromycin (MABE343, 1:100; Merck Millipore) for 45 min. After 3 wash steps in FACS buffer, cells were incubated in secondary antibodies donkey- α -mouse-cy5 (715-175-150, 1:200; Jackson ImmunoResearch) and goat- α -rabbit-Alexa594 (A-11012, 1:200; Invitrogen) for 45 min at room temperature in FACS buffer. Cells were washed

twice, resuspended in PBS + 1% paraformaldehyde and analyzed on the FACSCanto II (BD Biosciences).

AP-MS. Protein samples were separated by SDS-PAGE, and stained with Coomassie colloidal blue (Bio-Rad). After a short run, the entire sample between running fronts was sliced out and reduced, alkylated, and digested as previously described (43). Digests were subjected to nLC-MS/MS analysis using an Agilent 1290 Infinity UHPLC system (Agilent) coupled to an Orbitrap Q Exactive Plus mass spectrometer (Thermo Scientific). Dried peptides were reconstituted in 10% formic acid (FA) and delivered to a trap column (Dr Maisch Reprosil C18, 3 μ m, 2 cm $\times$ 100 μ m) at 5 μ L/min with solvent A (0.1% FA in water). Next, peptides were chromatographically separated onto an analytical column (Agilent Poroshell EC-C18, 2.7 μ m, 50 cm $\times$ 75 μ m) at 300 nL/min, as previously described (44). The gradient was as follows: 13–40% solvent B (0.1% FA in 80% Acetonitrile) in 65 min, to 100% solvent B in 3 min, 1 min of 100% solvent B, and finally equilibration of the chromatographic columns with 100% solvent A for the following 10 min before injection of the next sample. Total analysis time was 90 min. The eluent was sprayed via distal coated emitter tips butt-connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS. Full-scan MS spectra (from m/z 375 to 1600) were acquired in the Orbitrap with a resolution of 35,000 at m/z 400. The 10 most intense ions within the survey scan were selected for HCD fragmentation with normalized collision energy set to 25%. The MS/MS AGC target value was set to 5e4 with a maximum ion injection time of 120 ms. Dynamic exclusion was set to 12 s. Each raw data file recorded by the mass spectrometer was processed and quantified with Proteome Discoverer (version 2.1, Thermo Scientific), and searched against a Swissprot human database (version 2016_03, 20,199 sequences) supplemented with the Aichivirus polyprotein sequence (Uniprot id A0A2S1JL36) using Mascot software (version 2.5.1, Matrix Science). Trypsin/P was chosen as the protease, cysteine carbamidomethylation was selected as fixed modification, and oxidation of methionine as variable modifications. Precursor and fragments mass tolerance were set to 20 ppm and 0.05 Da, respectively. Proteins were further filtered for contaminants and highly abundant proteins (such as keratins, tubulins, and ribosomal proteins), number of unique peptides (> 0), and number of peptides (> 1). All peptide-spectrum matches (PSM) were validated with 5% false discovery rate using Percolator (45). Only PSMs with a minimum length of 6 amino acids were kept. To discriminate bona fide protein interactors of AiVL from the background, we further analyzed the hits by SAINT scoring (46).

Immunoprecipitation of flag-tagged eIF2B β . HeLa-R19-eIF2B β -3Xflag were transfected with plasmids encoding the indicated EGFP-tagged proteins using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. Transfection medium was replaced by fresh medium 24 hrs post transfection, and cells were allowed to express the

protein of interest for a total of 48 hours. For each sample, 3.0×10^7 cells were lysed in 500 μ L lysis buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X100, protease inhibitors [Roche], phosphatase inhibitors [Sigma-Aldrich]) for 15 min on ice. After spinning down to remove cell debris, 10 μ L magnetic anti-Flag Beads (Sigma-Aldrich) was added to the supernatants and incubated rotating for 2 hours at 4 °C. Beads were then washed four times in wash buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X100), and resuspended in 1X LSB. If indicated (See Fig. 5), the third wash step was done for 15 min in standard wash buffer, or high salt wash buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 500 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X100) to dissociate eIF2 from eIF2B. Samples were then separated by SDS-PAGE and analyzed by Western blotting using the indicated antibodies.

Immunoprecipitation of TAP-tagged AiVL. 1.5×10^7 HEK cells were transfected with plasmid encoding the indicated TAP-tagged protein using PEI transfection reagents as described previously (47). 24 hrs post transfection, cells were harvested and lysed in 500 μ L lysis buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X100, protease inhibitors [Roche]). 20 μ L S-beads was added to each sample, and incubated rotating for 2 hours at 4 °C. Beads were washed twice in wash buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X100), once in 3C^{pro} cleavage buffer (HRV 3C Protease Solution Kit; Thermo Scientific), and resuspended in 250 μ L 3C^{pro} cleavage buffer. 3C^{pro} was then added to proteolytically release proteins from the S-beads rotating overnight at 4 °C. Supernatants were collected and transferred to new tubes. 10 μ L Strep-Tactin beads (IBA) was added to each sample and incubated rotating for 1 hour at 4 °C. Beads were washed three times in wash buffer and subsequently resuspended in 1X LSB. Samples were then separated by SDS-PAGE and analyzed by Western blotting using the indicated antibodies.

Single-step growth curve. Vero cells were infected with AiV at an MOI of 10. After 1 hr, the cells were washed three times in PBS to remove excess virus. At 0, 2, 4, 6, 8, 10, or 16 hrs pi., samples were freeze-thawed three times to release intracellular virus. The total amount of infectious virus in each sample was quantified by end-point dilution assays.

Phylogenetic analysis of KoVL proteins. KoVL protein sequences (AiVL, NP_047200.1; M-KoVL, YP_004782207.1; C-KoVL, AHK60486.1; R-KoVL, AIK67137.1; P-KoVL, YP_002473934.1; F-KoVL, AGU62946.1; B-KoVL, NP_859018.1) were aligned in MEGAX software (48) using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) method. A phylogenetic tree was created by the Maximum Likelihood Method, using a JTT model (49), and a site coverage cut-off of 99%. The final dataset included 155 positions.

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Supplementary Figures

< Figure S1: IFA analysis of SG inhibition by the full panel of AiVL point mutants. The indicated EGFP-tagged AiVL mutant proteins were transiently overexpressed in HeLa cells. Next day, cells were treated with 250 μ M arsenite for 1 hr, and SG formation was analyzed by IFA using antibodies directed against SG markers G3BP1 and eIF3.

Figure S2: Transient AiVL overexpression yields high concentrations of soluble protein. (A) Flowcytometry analysis of HeLa cells transiently overexpressing the indicated proteins. (B) In parallel, cells were lysed in lysis buffer, and cell debris was spun down. In the resulting samples, containing an insoluble pellet and a soluble supernatant fraction, EGFP content was visualized under UV-light.

Chapter 7

General Discussion

The Picornaviridae are a large virus family consisting of 47 genera and 110 species and amongst these viruses are several human and veterinary pathogens. Viral infection is recognized by the host and triggers an antiviral response, which is centered around the production of type I interferons and the antiviral effects of interferon stimulated genes (ISGs). Meanwhile, viruses try to evade the antiviral effects of this response. Picornaviruses are known to predominantly depend on their security proteins (2A and L) to evade antiviral responses, although many of the underlying molecular mechanisms remain to be elucidated. In this thesis, we studied how several picornaviruses evade the induction of type I interferons and the antiviral activities of the ISG PKR. PKR acts as a dsRNA sensor and activator of the cellular stress response. In chapter 2-4 we studied the mechanisms employed by foot-and-mouth disease virus to evade the induction of type I interferons and the cellular stress response. In chapter 5 we investigated how enteroviruses suppress both antiviral pathways and in chapter 6 we explored how kobuviruses evade the cellular stress response. Here, the main findings are summarized and common themes in the modes of action of the different picornavirus security proteins are discussed.

I. Summary

In chapter 2, we investigated whether and how members of the *Aphthovirus* genus suppress the cellular stress response. We found that FMDV efficiently suppresses stress granule (SG) formation, which we found to be dependent on the catalytic activity of the Leader protease (L^{pro}). We generated chimeric EMCVs that encoded either FMDV L^{pro} or ERAV L^{pro} (FMDV's closest relative), to demonstrate that L^{pro} does not suppress PKR signaling but affects SG formation. We also demonstrated that L^{pro} cleaves the SG scaffolding protein G3BP1 and G3BP2, and we showed that G3BP1 cleavage occurs upon FMDV infection.

In chapter 3, we identified FMDV L^{pro} as a deISGylase and showed that L^{pro} has very little deubiquitinase activity. We demonstrate this preference of L^{pro} for ISG15 *in vitro*, but also in infected cells. Furthermore, we demonstrate that L^{pro} is a unique deISGylase, leaving a Gly-Gly modification on the lysine of target proteins, whereas canonical deISGylases hydrolyze the isopeptide linkage between ISG15 and the lysine of the target protein. The presence of uniquely modified lysines in proteins from FMDV-infected cells can potentially be used to develop new FMDV detection strategies, which can efficiently distinguish between infected and vaccinated animals.

The findings in chapter 3 also initiated the research described in chapter 4. FMDV suppresses the production of type I interferons by suppressing RLR signaling but also by directly affecting translation of IFN- α/β . FMDV L^{pro} inhibits cap-dependent translation

by cleaving eIF4G and this likely affects IFN- α/β translation. FMDV also requires L^{pro} to suppress RLR signaling but the underlying mechanism remains to be fully elucidated as several mechanisms have been proposed over the years. L^{pro} has been suggested to degrade the transcription factors IRF3, IRF7 and NF κ B (1, 2). Additionally, L^{pro} 's DUB activity has been suggested to interfere with RLR signaling (3). While L^{pro} does have DUB activity upon overexpression, we found no evidence of significant DUB activity during viral infection (chapter 3). In chapter 4, we investigated whether the deISGylase activity of L^{pro} contributes to the suppression of RLR signaling or whether alternative mechanisms contribute to this ability of L^{pro} . First, we demonstrated that L^{pro} cleaves the RLR signaling proteins TBK1 and MAVS. Introduction of specific mutations in L^{pro} subsequently allowed us to identify amino acids that are essential for either L^{pro} 's deISGylase/DUB activity or its proteolytic activity towards RLR signaling proteins. We demonstrated that a L^{pro} mutant that is severely impaired in its deISGylase activity can still suppress IFN- β gene transcription, while a L^{pro} mutant that is severely impaired in the cleavage and degradation of RLR signaling proteins cannot. Our combined observations suggest that L^{pro} suppresses RLR signaling independently from its deISGylase activity and point to a role for the cleavage of RLR signaling proteins.

In chapter 5, we focused on members of the *Enterovirus* genus and investigated their ability to evade the induction of IFN- α/β gene expression and the cellular stress response. The two enterovirus proteases (i.e. $2A^{\text{pro}}$ and $3C^{\text{pro}}$) have been suggested to help the viruses evade antiviral responses. We compared the ability of individual enterovirus proteases of representative members of the enterovirus species EV-A to EV-D to interfere with the induction of IFN- α/β gene expression as well as cellular stress response activation. This comprehensive analysis revealed that enterovirus $2A^{\text{pro}}$ of multiple enteroviruses is able to suppress IFN- α/β gene expression and inhibit SG formation. $2A^{\text{pro}}$ inhibits SG formation but does not affect cellular stress response signaling (e.g. counteract the phosphorylation of PKR or eIF2 α), suggesting that $2A^{\text{pro}}$ targets SG assembly. SGs themselves have been suggested to act as a signaling platform that is critical for the induction of IFN- α/β expression (4, 5). Therefore, we investigated whether the effects of $2A^{\text{pro}}$ on RLR signaling depend on $2A^{\text{pro}}$'s ability to counteract SG assembly. Using cells that are deficient in SG formation (i.e. PKR k.o. MEFs and G3BP1/G3BP2 double k.o. HeLa cells), we demonstrated that the inability of cells to form SGs reduces IFN- α/β gene expression ~10-fold. Thus, we provide evidence that SG formation contributes to, but is not critical for, RLR signaling. In addition, we demonstrated that $2A^{\text{pro}}$ suppresses IFN- α/β gene expression ~500-fold, suggesting that $2A^{\text{pro}}$ has additional mechanisms to directly target RLR signaling.

In chapter 6 we investigated how members of the *Kobuvirus* genus evade the cellular stress response. Thus far, viruses counteract the stress response by directly antagonizing SG

Figure 1: The RLR signaling pathway is targeted by multiple picornavirus proteins. This schematic representation of the RLR signaling pathway has been introduced in chapter 1. This version indicates which signaling components are targeted by enterovirus 2A^{pro}, enterovirus 3C^{pro}, FMDV L^{pro} and the cardiovirus L protein. This figure was made with BioRender.

formation, or by preventing and/or reversing eIF2 phosphorylation. Aichivirus, through the activity of L, allows ongoing translation in the presence of high levels of p-eIF2. Aichivirus L binds eIF2B and this prevents association of p-eIF2 to eIF2B. Hereby, p-eIF2 no longer interferes with the interaction between non-phosphorylated eIF2 and eIF2B, and translation can continue. Mouse kobuvirus L, which is the closest relative of aichivirus L, was the only other kobuvirus L that rescued translation upon high levels of p-eIF2. The inability of other kobuviruses leads to interfere with the cellular stress response suggests that this ability was a recent gain-of-function event and that kobuvirus leader likely has another primary function.

Figures 1 and 2 provide a schematic representation of the RLR signaling cascade (fig. 1) and the cellular stress response (fig. 2), and indicate how the security proteins studied in this thesis counteract these antiviral responses. In the experiments described in this thesis, we frequently

Figure 2: The cellular stress response is targeted by multiple picornavirus proteins. This schematic representation of the cellular stress response has been introduced in chapter 1. This version indicates where in the pathway enterovirus 2A^{pro}, enterovirus 3C^{pro}, FMDV L^{pro}, cardiovirus L, and Aichivirus L counteract the response. This figure was made with BioRender.

made use of the cardiovirus EMCV and an EMCV mutant virus (EMCV-L^{Zn}) that carries inactivating mutations in its antagonist of the innate antiviral responses (EMCV-L^{Zn}) as positive and negative controls. Therefore, the mechanisms of cardiovirus L have been included in figures 1 and 2. Cardiovirus L is a well described antagonist of RLR signaling as well as the cellular stress response. Cardiovirus L efficiently suppresses RLR signaling by preventing or counteracting IRF3 phosphorylation, dimerization and translocation to the nucleus (collectively referred to as IRF3 activation) (6–8). In addition, cardiovirus L inhibits the cellular stress response at, at least, two stages. Cardiovirus L inhibits the phosphorylation of PKR (9, 10) and thereby prevents the downstream phosphorylation of eIF2 and the formation of SGs. L also prevents the formation of SGs upon addition of sodium arsenite (10, 11), although under these conditions L fails to inhibit eIF2 phosphorylation (10). Thus, L inhibits PKR phosphorylation and L counteracts the stress response at a step between eIF2 phosphorylation and SG formation.

II. Common themes in the mechanisms used by picornavirus security proteins

The picornavirus security proteins are the least conserved amongst the picornavirus proteins and the mechanisms they employ are incredibly diverse. We identified several intriguing parallels in the mechanisms used by the different security proteins, which could indicate specific vulnerable sites in the antiviral responses that can be exploited by viruses. In this section these common themes and their implications are discussed.

1. Counteracting SG assembly; a matter of targeting G3BP1 and eIF4G?

In chapter 2, we describe that FMDV suppresses SG formation via its leader protease (L^{pro}), and show that suppression of SG formation depends on L^{pro} 's catalytic activity. We also show that L^{pro} cleaves the SG scaffolding proteins G3BP1 and G3BP2. We do not demonstrate a causal relationship between SG suppression and G3BP1 and G3BP2 cleavage, but a causal relationship between G3BP1 cleavage and SG suppression has been suggested for a number of enteroviruses (12–14). However, in chapter 5 we showed that the cleavage of G3BP1 by enterovirus 3C pro only modestly reduced SG formation. Instead, we and others (chapter 5 and (15)) demonstrated that enterovirus 2A pro very efficiently suppressed SG formation. Thus far, enterovirus 2A pro has not been demonstrated to cleave any known SG scaffolding proteins and thus it is debatable whether there is a causal relationship between G3BP1 cleavage and inhibition of SG formation.

Importantly, in chapter 5 we also showed that both G3BP1 and G3BP2 need to be depleted to abolish SG formation. In contrast to enterovirus 3C pro , FMDV L^{pro} cleaves both G3BP1 and G3BP2, and thus it remains plausible that the L^{pro} -induced cleavages suppress SG formation. Enterovirus 3C pro only targets G3BP1 and while this does reduce the number of SGs, it is insufficient to completely prevent SG formation. Without evidence of a causal relationship between the L^{pro} -mediated cleavage of G3BP1 and G3BP2 and the ability of L^{pro} to suppress SG formation – for example by determining whether L^{pro} suppresses SG formation in cells expressing cleavage resistant G3BP1 and G3BP2 – we should also consider alternative mechanisms via which L^{pro} could suppress SG formation.

It was recently suggested that enterovirus 2A pro breaks a newly identified interaction between G3BP1 and eIF4G (16). Breaking of this interaction, which was shown to depend on 2A pro 's catalytic activity, was suggested to be the mechanism via which 2A pro inhibits SG formation. One would expect the cleavage of eIF4G by 2A pro to cause the loss of the eIF4G-G3BP1 interaction. Counterintuitively, Yang *et al.* showed that 2A pro also disrupts the interaction between G3BP1 and a 'cleavage resistant' eIF4G (eIF4G G689E) and 2A pro continued to suppress SG formation under these conditions (16). EMCV L, which lacks

catalytic activity, was also shown to disrupt the eIF4G-G3BP1 interaction (16). This could explain the observation that cardiovirus L can counteract sodium arsenite-induced SG formation, but not sodium arsenite-induced eIF2 phosphorylation (10, 11). Collectively, these observations support the idea that both 2A pro and cardio L target SG assembly. This hypothesis may be investigated further with the use of pateamine A, an inhibitor of eIF4A and thus a molecule that blocks translation initiation independently of p-eIF2 (17, 18). At the present time, the molecular mechanism(s) underlying the suppression of SG formation by enterovirus 2A pro , cardiovirus L and FMDV L^{pro} remain only partially understood. The available data hint to a possible role for targeting the interaction between eIF4G and G3BP1 by multiple picornavirus security proteins, but this requires further investigation.

To fully comprehend the strategies employed by viruses to counteract SG formation, one needs to understand the driving forces of SG formation. In recent years, several unbiased large-scale analyses have been performed to investigate the protein and RNA composition of "core" SGs (19, 20). In these studies, G3BP1 was precipitated and the precipitated complexes were subsequently analyzed using mass spectrometry or RNA-seq. A recent study combined data of a genome-wide RNAi screen with a comprehensive bioinformatics analysis of the previous SG datasets to generate a "core SG network" consisting of 36 protein (21). This network includes most proteins previously claimed to be essential for SG formation (e.g. TIA-1 and G3BPs), but notably lacks eIF4G. Out of the 36 proteins within the core SG network, only the double knock-out of G3BP1 and G3BP2 resulted in a cell line completely devoid of SG formation upon treatment with sodium arsenite, which is consistent with the results described in chapter 5 of this thesis. The high "betweenness centrality" of the G3BPs in the core SG network suggested that they are an essential driving force for the formation of SGs, at least during sodium arsenite-induced cellular stress. Subsequent analysis showed that G3BP1 and G3BP2 can undergo RNA-dependent phase separation. The ability of G3BP1 and G3BP2 to undergo phase separation was dependent on their RNA binding domain (RBD), which itself consists of an RNA recognition motif (RRM) and the intrinsically disordered region 3 (IDR3). Simultaneously, their NTF2-like domain was found to be important for their ability to dimerize. The ability of G3BPs to dimerize as well as their ability to undergo phase separation were identified as essential drivers underlying SG formation. Meanwhile, the additional proteins of the core SG network were found to lower the thresholds of G3BP phase separation and thereby positively influence SG formation. These new insights into the biophysical processes underlying SG formation highlight the central roles of G3BP1 and G3BP2 and support the previously discussed mechanisms via which enterovirus 2A pro , cardiovirus L and FMDV L^{pro} could counteract SG formation.

Similarly, one must understand the “requirements” for an RNA molecule to be incorporated into SGs. RNAseq analysis of the SG “core” revealed that SGs primarily consist of mRNA; 78% of the RNA from U-2 OS cell derived SGs is mRNA, this further increases to 95% when yeast derived SGs are analyzed (20). Meanwhile, a U-2 OS cell transcriptome consists of only 17% mRNA, while the other 83% is made up of various ncRNA molecules. Furthermore, the mRNAs that are enriched in the SG core are not the overly abundant mRNAs (e.g. actin), have less ribosome density and are rather long (average length is 7.1 kb) (20). One should also investigate a possible role for eIFs bound to mRNA in the recruitment of mRNAs to SGs. Several eIFs have been found to localize to SGs. The eIFs found in SGs are reminiscent of the 48S pre-initiation complexes, with the notable exception of eIF2. These eIFs are suggested to be coordinately recruited to SGs, as they remain bound to mRNAs that are recruited to SGs (22). Intriguingly, eIF4E carrying an inactivating mutation in its eIF4G/eIF4E-BP interaction domain failed to localize to SGs, while wt eIF4E does localize to SGs (23, 24). mRNAs bound by the mutant eIF4E lack all other eIFs and fail to recruit the pre-initiation complex. Failure of these eIF4E-mRNA complexes to localize to SGs could suggest that mRNAs lacking certain eIFs are not incorporated in SGs.

Cleavage of eIF4G by 2A^{pro} separates the eIF4E-interaction domain (i.e. eIF4Gs N-terminus) from the domain that interacts with further eIFs and thus facilitates pre-initiation complex recruitment (eIF4Gs C-terminus). An mRNP carrying just eIF4E and the eIF4G N-terminal cleavage fragment lacks most eIFs and is nearly identical to a mRNP containing the eIF4E interaction domain mutant discussed above. Which eIFs need to be bound to a mRNA for said mRNAs to be recruited to SGs unfortunately remains incompletely understood. Therefore, it remains unclear whether mRNPs that contain the eIF4G N-terminal cleavage product rather than full length eIF4G, and thus lack other eIFs such as eIF3 and eIF4A, are recruited to SGs. Importantly, enterovirus 2A^{pro} and FMDV L^{pro} cleave eIF4GI and eIF4GII at nearly the same place, the respective cleavage sites are located just a few residues apart (25). Thus, one would suggest that eIF4G cleavage by either protease has a similar effect on SG formation. It is worthwhile to investigate whether FMDV L^{pro}'s ability to cleave eIF4G contributes to its ability to suppress SG formation. This may ultimately shed light on the possible causality between eIF4G cleavage and counteracting SG assembly.

Of note, we and others observed that expression of enterovirus 2A^{pro} can induce formation of ‘atypical’ SGs that contain some, but not all, *bona fide* SG marker proteins (chapter 5 and (15, 26)). The atypical SGs were suggested to be induced by the sudden surplus of non-translating mRNPs, as a result of eIF4G cleavage (26). As infection continues, the atypical SGs dissolve and the cells subsequently fail to form SGs. Expression of FMDV or ERAV L^{pro} resulted in the accumulation of similar small SGs that subsequently disappeared (chapter 2). A scenario where cleavage of eIF4G first induces formation of atypical SGs, and where

eIF4G subsequently plays a role in inhibiting the formation of *bona fide* SGs, illustrates the complexity of virus-host interactions. A recent 2A^{pro} interactome analysis identified the eIF3 complex as an important interaction partner of 2A^{pro} (27). Whether and how the interaction between 2A^{pro} and eIF3 contributes to the ability of 2A^{pro} to evade SG formation, or promotes the formation of atypical SGs, remains to be determined. The interaction between 2A^{pro} and eIF3 should be considered when investigating how 2A^{pro} suppresses SG formation.

2. Promoting viral translation in the presence of phosphorylated eIF2

Canonical translation is inhibited by the phosphorylation of eIF2. This could also affect viral translation and thus phosphorylation of eIF2 is potentially antiviral. To successfully replicate, viruses overcome the negative effects of phosphorylation of eIF2. Many viruses (i.a. herpes simplex virus, respiratory syncytial virus, human immunodeficiency virus and transmissible gastroenteritis virus) rescue viral translation by preventing or reversing the phosphorylation of eIF2 (28–31). Another strategy is to ensure that viral translation is resistant to the inhibitory effects of p-eIF2.

Several viruses have evolved noncanonical translation mechanisms that are independent of (most of) the eukaryotic translation initiation factors, which ensures viral translation to continue despite the phosphorylation of eIF2. One example is the translation of the subgenomic RNA (sgRNA) molecule of alphaviruses that is generated during genome replication, which occurs independent of translation initiation factors eIF2 and eIF4A (32–35) during the later stages of infection, when eIF2 is phosphorylated. Translation of sgRNA in the presence of p-eIF2 was found to depend on a hairpin structure in the coding region, known as the DLP (35). The DLP is located downstream of the initiator AUG and was suggested to stall a scanning ribosome exactly at this codon, thereby facilitating translation initiation independently of eIF2(35). Another example is the Cricket paralysis virus intergenic region IRES (CrPV IRES), which facilitates translation independently of all translation initiation factors. The 40S ribosomal subunit can directly interact with the CrPV IRES, and this signals for recruitment of the 60S subunit to facilitate 80S formation (36, 37). The generated 80S ribosome directly facilitates translation ‘initiation’ from a non-AUG codon via translation elongation factors 1A and 2 (38). Translation from the CrPV IRES completely bypasses the intricate process of eukaryotic translation initiation and is thereby resistant to inhibition by p-eIF2.

Picornavirus translation is also directed from an IRES, in fact the poliovirus and EMCV IRESes were amongst the first discovered IRES structures. Translation initiation from the picornavirus IRESs is slightly more complex than CrPV IRES-driven translation. Picornavirus translation starts from a downstream AUG instead of directly at the IRES,

this requires the ribosome to assemble at the IRES structure and subsequently scan for the start codon. Thus far, all identified picornavirus IRESes need most translation initiation factors, including eIF2, to direct translation *in vitro* (39–42). Despite the need for eIF2 in *in vitro* IRES-mediated translation, picornaviruses efficiently translate and replicate *in vivo* and *in cellulo* under conditions of cellular stress. This suggests that they have evolved mechanisms either to prevent the phosphorylation of eIF2 or that viral translation is resistant to the inhibitory effects of p-eIF2.

During enterovirus infection PKR and eIF2 are phosphorylated (13, 15, 43, 44), suggesting that enteroviruses facilitate viral translation despite high levels of p-eIF2. Indeed, enterovirus translation was reported to be resistant to the inhibiting effects of p-eIF2 (43, 45). Treatment of cells with sodium arsenite, applying heat shock, or addition of an eIF2 α phosphatase-inhibitor increased the level of p-eIF2 without significantly affecting poliovirus translation at the mid-to-late stages of infection. Translation of CVB3 was found to be similarly resistant to p-eIF2, demonstrating that this ability is likely to be conserved amongst enteroviruses (45). Interestingly, the earliest viral translation (at 2 hours post infection) was affected by the artificial induction of eIF2 phosphorylation, suggesting that enteroviruses switch from canonical translation to a p-eIF2 resistant alternative mode of translation. In contrast to viral translation, expression of a IRES-containing luciferase reporter construct is inhibited by p-eIF2 (43, 45). This observation illustrates that translation from a type II IRES is only resistant to p-eIF2 in the context of viral infection, which suggests that a viral product mediates the switch from canonical to p-eIF2 resistant translation. Because the early viral translation is affected by p-eIF2, it is estimated that this switch occurs around 2 hours post infection, which coincides with the accumulation of the viral proteins in the cell (45). Enteroviral proteases 2A^{pro} and 3C^{pro} have both been implicated to mediate the switch from p-eIF2 sensitive to p-eIF2 resistant viral translation.

Enterovirus 3C^{pro} targets eIF5B (46) and this has been suggested to facilitate the switch to p-eIF2 resistant translation (45). eIF5B is a homologue of prokaryotic IF2 that facilitates 80S assembly and stabilizes the initiator tRNA^{Met} when eIF2 has been released. Ectopic overexpression of wt eIF5B stimulated poliovirus translation in the presence of sodium arsenite. Interestingly, ectopic expression of cleavage resistant eIF5B (eIF5B Q478E) impaired viral translation at 6 hours post infection, which suggests that efficient viral translation requires the cleavage of eIF5B (45). In agreement with this observation, the eIF5B C-terminal cleavage fragment was shown to positively influence IRES translation (~2-fold increase) in the presence of sodium arsenite (45). Eukaryotic eIF5B contains a large N-terminal extension that is absent in its prokaryotic (IF2) and archaeal (aIF5B) homologues, and it has been shown that full length as well as N-terminally truncated yeast eIF5B can interact with initiator tRNA^{Met}, albeit less efficiently than eIF2 (47). White *et.al.*

suggested that the C-terminal cleavage product of eIF5B behaves more like prokaryotic IF2 and their data clearly demonstrate that cleavage of eIF5B by 3C^{pro} is needed to facilitate p-eIF2 resistant translation (45).

Hepatitis C virus (HCV) and classical swine fever virus (CSFV) were reported to exploit full length eIF5B to overcome the negative effects of p-eIF2 on viral translation (48, 49). Translation initiation from both the HCV and CSFV IRES is rather straightforward; the 40S subunit directly interacts with the IRES, in a mechanism similar to the CrPV IRES (50, 51). In the case of the HCV and CSFV IRES, translation initiation does depend on the ternary complex (eIF2-GTP-tRNA^{Met}) and eIF3 and thus viral translation, theoretically, is sensitive to the phosphorylation of eIF2 (51–54). However, for both IRESes it has been demonstrated that they allow functional replacement of eIF2 by eIF5B, in particular under conditions where eIF2 is phosphorylated (48, 49). The possibility to hijack eIF5B to ensure viral translation despite the phosphorylation of eIF2 may illustrate a vulnerable site in the hosts antiviral defenses that many viruses, including enteroviruses, take advantage off.

Enterovirus 2A^{pro} was also suggested to facilitate the switch to a p-eIF2 resistant mode of viral translation (43). In sodium-arsenite treated cells, ectopic overexpression of 2A^{pro} rescued IRES-mediated translation, while ectopic overexpression of the other viral proteins, including 3C^{pro}, did not. The ability of 2A^{pro} to rescue translation upon high levels of p-eIF2 correlates with the catalytic activity of 2A^{pro}, as determined by the efficiency of eIF4G cleavage. Expression of the eIF4G N- and C-terminal cleavage products was not sufficient to rescue viral translation, suggesting that 2A^{pro} cleaves another unidentified host protein to switch from canonical translation to a p-eIF2 resistant mode of translation. Whether enterovirus 2A^{pro} and 3C^{pro} act in a synchronized manner to facilitate viral translation when eIF2 is phosphorylated has thus far not been explored.

FMDV L^{pro} was also reported to facilitate p-eIF2 resistant IRES-driven translation. L^{pro} supports translation in the presence of sodium arsenite from both type I and type II IRES structures, and this ability coincides with cleavage of eIF4G by L^{pro} (55). FMDV L^{pro} and enterovirus 2A^{pro} are structurally different enzymes but share many functions; both cleave translation initiation factor eIF4G (56–58), both reduce type I interferon gene expression (chapters 4, 5 and (6, 59, 60)), and both have been implicated in the suppression of SG formation (chapters 2, 5 and (15)). Thus far there is evidence that the catalytic activity of both viral proteases facilitates the switch in translation mechanism, but the exact proteolytic target remains to be elucidated. Furthermore, there is evidence that cardiovirus translation continues despite high levels of p-eIF2, although this has not been linked to a specific viral protein (61).

Meanwhile, we uncovered that Aichi L can counteract the cellular stress response at its core. Aichi L interferes with the p-eIF2 • eIF2B interaction, thereby allowing the continued formation of new ternary complex despite the presence of high levels of p-eIF2. Viral antagonists of the cellular stress response identified thus far either prevented or reversed the phosphorylation of eIF2 (28–31). Aichi L can antagonize the cellular stress response and rescue translation, irrespective of the nature of the stressor and stress severity. Therefore, this is arguably one of the most efficient ways to block the cellular stress response described to date. This novel mechanism of cellular stress response antagonism has been simultaneously identified for another viral protein, the accessory protein 10 (AcP10) of beluga whale coronavirus (BW-CoV) (62). Both viral proteins appear to have a different primary function and acquired stress response antagonism via recent gain-of-function mutations. The acquisition of a novel stress response antagonism strategy by a picornavirus and coronavirus is an interesting example of convergent evolution.

These collective observations suggest that rescuing viral translation from the inhibitory effects of phosphorylated eIF2 may be a strategy commonly employed by many picornaviruses, albeit via various underlying molecular mechanisms. The mechanisms underlying enterovirus 2A^{pro} /3C^{pro} and FMDV L^{pro}-mediated p-eIF2-resistant IRES-driven translation remain to be elucidated, but clearly differ from the mechanism used by aichivirus L. 2A^{pro}, 3C^{pro}, and L^{pro} have been suggested to rely on their catalytic activity to restore IRES translation. Aichivirus L lacks catalytic activity and instead relies on a protein-protein interaction between itself and eIF2B to circumvent the inhibitory effects of the cellular stress response. Meanwhile, the viral protein involved in facilitating non-canonical translation in cardiovirus infection remains to be identified. As such, it is currently unknown whether continued translation in cardiovirus-infected cells depends on the catalytic activity of 3C^{pro}, or whether cardioviruses employ a strategy that is perhaps more similar to that of aichivirus and mouse kobuvirus.

3. Connection between nucleocytoplasmic trafficking disorder and impaired RLR signaling?

In chapter 5, we studied whether and how enterovirus proteases affect RLR signaling. We showed that enterovirus 2A^{pro}, via its catalytic activity, suppresses RLR signaling. 2A^{pro} is known to cleave RLR signaling proteins (6, 60), but a causal relationship between the cleavage of RLR signaling proteins and the suppressed induction of IFN-β mRNA remains to be demonstrated. We showed that 2A^{pro}'s catalytic activity is needed to suppress IFN-β mRNA, but we did not specify whether 2A^{pro}'s catalytic activity towards RLR signaling proteins is a contributing factor (chapter 5). While future research will undoubtedly aim to demonstrate a causal relationship between cleavage of RLR signaling proteins and suppression of IFN-α/β mRNA induction, we cannot exclude the contribution of alternative mechanisms at this point.

One alternative mechanism involves the ability of 2A^{pro} to induce a so-called nucleocytoplasmic trafficking disorder (NCTD). 2A^{pro} of several different enteroviruses cleaves nucleoporins, which results in the collapse of nuclear pores (63–68). As a result, active nucleocytoplasmic transport is disturbed and proteins can migrate in and out of the nucleus via passive diffusion. Cardiovirus L also suppresses the induction of IFN-α/β mRNA and it also induces NCTD, albeit via a different underlying molecular mechanism. L interacts with RAN-GTPase (69) to localize close to the nuclear envelope, where it facilitates the phosphorylation of nucleoporins (NUPs) (70, 71). The resulting negative charge on the NUPs destabilizes the nuclear pore complex and results in impaired functioning of the nuclear pore. It was previously demonstrated that MAPK kinase inhibitors inhibit NUP hyperphosphorylation, implicating cellular MAPK kinases in this phenomenon (72). L was recently reported to interact with, and prevent the inactivation of the cellular p90-ribosomal S6 kinase (RSK) (73). RSK belongs to the MAPK family of kinases and thus is likely the kinase that phosphorylates the nucleoporins.

Collapse of the nuclear pore and the subsequent inhibition of active transport in and out of the nucleus can affect the regulated translocation of IRF3, IRF7 and NFκB and can thereby impair the transcription of IFN-α/β mRNA. Strikingly, the NCTD that is triggered by cardiovirus L did not prevent IRF3 from localizing to the nucleus (74). However, IRF3 lacking its nuclear translocation signal (NLS) also localized to the nucleus of cardiovirus-infected cells, while this IRF3-ΔNLS did not localize to the nucleus upon poly(I:C) stimulation (74), suggesting that nuclear localization of IRF3 observed during infection is due to the NCTD rather than its activation. Similarly, enterovirus 2A^{pro} induced the relocalization of GFP-NLS as well as well-known nuclear proteins (i.e. Sam68, nucleolin and La autoantigen) to the cytosol (68, 75). Enterovirus 2A^{pro} and cardiovirus L were also shown to have a general effect on regulated nuclear transport; expression of either security protein interfered with overall nuclear import (by affecting the transportin 1, transportin 3 and importin-α/β pathways) as well as nuclear export (76, 77). It is worth emphasizing that the translocation of IRF3, NFκB and STAT1 to the nucleus depends on the importin-α/β transporters (78–80). Other viruses (i.e. hepatitis C virus and Ebola) are known to counteract IFN-α/β and ISG expression by inhibiting the importin-α/β-dependent nuclear import pathways (78, 81). These collective observations strongly suggest that NCTD induction by cardiovirus L and enterovirus 2A^{pro} could affect the nuclear import of IRF3, IRF7 and NFκB and thus contribute to the suppression of IFN-α/β expression.

The effect of an impairment in nucleocytoplasmic transport on innate antiviral signaling has also been studied outside of the context of viral infection. It was recently demonstrated that NUP93 deficient cells have reduced expression of IFN-β and the pro-inflammatory cytokine CXCL10 (82). NUP93 is essential for the formation of the nuclear pore complex. NUP93s

C-terminal part stabilizes the initial NUP53-NUP155 interaction and thereby contributes to the structural formation of a pore in the nuclear membrane. Meanwhile, its N-terminal domain recruits NUP62 and thereby transforms the structural pore into a functional NPC that facilitates nuclear import and export (83). In NUP93 deficient cells, IRF3 failed to translocate to the nucleus and activation of both IRF3 and TBK1 was impaired (82), suggesting that nuclear pore integrity can influence the RLR signaling cascade upstream of IRF3 translocation (i.e. TBK1 and IRF3 phosphorylation). Cardiovirus L as well as enterovirus 2A^{pro} target NUP62, the NUP that interacts with NUP93 to facilitate transport through the NPC. Both enterovirus 2A^{pro} and cardiovirus L were shown to reduce IRF3 phosphorylation and dimerization (6–8, 60, 84, 85). The observation that NUP93 depletion and the resulting disruption of the nuclear pore has an effect on the activation of both IRF3 and TBK1 could further support a role of NCTD in the suppression of IFN- α/β mRNA expression by cardiovirus L and enterovirus 2A^{pro}.

To confirm a causal relationship between NCTD and suppression of IFN- α/β mRNA expression, one would need to use CRISPR-cas9 technology to generate NCTD-resistant cells, i.e. cells where all 2A^{pro} cleavage sites in NUPs are mutated or cells where cardiovirus L can no longer induce hyperphosphorylation of NUPs. Unfortunately, the generation of such cell lines remains impossible. 2A^{pro} cleaves multiple NUPs and a complete understanding of which NUPs are cleaved at which site is currently lacking. The NUP cleavage sites are best described for HRV2 2A^{pro}, which cleaves NUP62 at six different positions (A103, G177, G201, G218, G247, A298) and NUP98 at two different positions (G374 and G552) (64, 66, 86), but the cleavage site(s) in NUP153 (66, 76) remain to be elucidated. Moreover, 2A^{pro} may cleave other NUPs as well, thereby hindering the generation of cells that are completely resistant to NCTD induction. The generation of cells resistant against cardio L-induced NCTD would be equally challenging. The hyperphosphorylation of NUPs that occurs during cardiovirus infection, which depends on L, was suggested to be analogous to NUP phosphorylation during prophase, the first stage of mitosis (70). Mutation of phosphorylation sites that possibly overlap with those normally used during cell division will most likely not result in a viable cell line. In conclusion, although the CRISPR-cas9 technology provides an exciting new horizon where it is possible to introduce mutations in endogenous genes, the generation of NCTD-resistant cell lines remains impossible for a variety of reasons.

Taken together, it remains to be elucidated exactly how enterovirus 2A^{pro} and cardiovirus L suppress RLR signaling. As discussed above there are strong indications that both security proteins rely on NCTD to inhibit the expression of IFN- α/β , but unfortunately it remains difficult to design the experiments needed to study this hypothesis. 2A^{pro} is known to cleave the RLR signaling proteins MDA5 and MAVS, while RLR signaling proteins remain intact during cardiovirus infection. The effects of the 2A^{pro}-induced cleavages of MDA5 and MAVS on IFN- α/β mRNA induction remain to be demonstrated, but cleavage of RLR signaling proteins

is commonly considered a strategy for viruses to counteract the expression of IFN- α/β . The timing of 2A^{pro} induced NCTD and 2A^{pro} induced cleavage of RLR signaling proteins varies. NCTD is observed as early as 2 hours post infection (63, 67), while MDA5 and MAVS cleavage by enterovirus 2A^{pro} is first detectable at 6-8 hours post infection (6, 60). The fast induction of NCTD could be considered as an additional argument that NCTD may be the main contributor to the early suppression of IFN- α/β expression. The cleavage of RLR proteins by enterovirus 2A^{pro} may well be a contributing factor to the continued suppression of IFN- α/β expression during the mid-to-late phase of enterovirus infection. Of note, cardiovirus infection does not result in cleavage of RLR proteins. Whether cardiovirus L utilizes additional mechanisms to suppress IFN- α/β expression during the mid-to-late phase of infection, remains to be established.

4. What is the role of the deISGylase activity of FMDV L^{pro}?

FMDV L^{pro} does not induce NCTD, yet it suppresses the induction of IFN- α/β (59). In chapter 4, we studied how FMDV L^{pro} suppresses RLR signaling and identified a correlation between the ability of L^{pro} to cleave RLR signaling proteins and L^{pro}'s ability to suppress IFN- β mRNA induction. L^{pro} also possesses deISGylase and, to a lesser extent, deubiquitinase (DUB) activity (chapter 3 and (3)) and these enzymatic activities are used by other viruses to suppress RLR signaling. In particular, the nidoviruses are known to suppress IFN- α/β transcription via the DUB/deISGylase activity of their papain-like protease (87, 88). It was suggested that L^{pro}'s DUB activity similarly contributes to suppression of RLR signaling (3). However, in chapter 3 we found no evidence of significant DUB activity of L^{pro} in infected cells, and in chapter 4 we suggest that FMDV L^{pro} suppresses IFN- α/β transcription independent from its deISGylase activity.

Our data demonstrate that L^{pro} can suppress IFN- β transcription and suggest that this ability relies on L^{pro}'s capacity to cleave RLR signaling proteins rather than its deISGylase activity, although a true causal relationship remains to be demonstrated. A recent report demonstrates that the deISGylase activity of L^{pro} contributes to viral spread *in vivo* (89). In agreement with our findings, impaired deISGylase activity of L^{pro} did not result in an increase in IFN- α/β or ISG mRNA in this study. How L^{pro}'s deISGylase activity benefits FMDV infection, both *in vivo* and *in cellulo*, is currently unknown. Our work has elucidated residues in L^{pro} that are essential for either its ability to cleave RLR signaling proteins or its deISGylase activity. Introduction of mutations in L^{pro} in the FMDV genome is feasible provided that L^{pro} can release itself from the polyprotein. This is illustrated by the previous successful generation of an FMDV strain carrying mutations in the SAP domain and an FMDV strain carrying a mutation affecting L^{pro}'s deISGylase activity (89, 90). This provides an opportunity to introduce the mutations we identified into the FMDV genome to study how L^{pro}'s deISGylase activity contributes to viral spread, possibly in an *in vivo* setting in livestock animals. Thus far, all data suggest that L^{pro}'s deISGylase activity may be needed

to combat one or several of the many other antiviral functions of ISG15 (reviewed in (91)), rather than contributing to the suppression of RLR signaling and thus the suppression of IFN- α/β expression.

In chapter 3, we also demonstrated that L^{pro} is a unique deISGylase, leaving a diglycylated-lysine on its substrates. The presence of these diglycylated lysines provides an interesting opportunity to investigate whether and how ISG15 regulates antiviral signaling. A specific monoclonal antibody suitable to enrich diglycylated lysine-containing peptides and proteins is available (92, 93). Previous applications of this antibody have been in the enrichment of diglycyl-lysine containing peptides in mass spectrometry analysis of ubiquitinated proteins, as tryptic digestion of ubiquitinated and ISGylated proteins results in the same diglycylated-lysine residues. A major drawback of these procedures has been the relatively late enrichment of modified peptides. There is the risk of missing modified proteins when the ubiquitin or ISG15 is lost during sample preparation. Additionally, all prior enrichment steps precipitate the total pool of a substrate protein, while only the small percentage of ubiquitin or ISG15-modified protein is of interest for the MS analysis. Expressing L^{pro} in an infected cell, by using EMCV-L^{pro}, has the advantage that proteins are ISGylated as a result of infection and the fragile ubiquitin- or ISG-modification is transformed into a stable diglycylated-lysine residue before cell lysis. Furthermore, we can directly enrich for those peptides carrying diglycylated-lysines, which will greatly improve the detection of modified proteins. This systematic approach will inform us of the (signaling) proteins that are modified with ISG15 upon viral infection, which has the potential to greatly increase our understanding of the function of protein ISGylation during infection.

III. Concluding remarks

In this thesis we studied how different picornavirus security proteins suppress or circumvent the activation of antiviral host responses. In the experimental chapters we provided new insight into the mechanisms used by FMDV, human enteroviruses and aichivirus to evade the induction of type I interferon or the antiviral properties of the cellular stress response. Here, we summarized our main findings and discussed commonly found tactics employed by different picornaviruses and their different security proteins. The analysis of the different mechanisms used to evade the antiviral responses revealed the possibility that multiple effects could be linked to a single action of a security protein. For example, the cleavage of eIF4G is known to ensure shut-off of cap-dependent (host) translation. Additionally, evidence is emerging that targeting of eIF4G could play a central role in the mechanisms used by security proteins to counteract SG formation. Similarly, the induction of NCTD by security proteins could have a direct effect on the RLR signaling pathway and thus on the expression of type I interferon and additional pro-inflammatory cytokines. The “network” of the different effector functions of a security protein, and whether and how these effects can be linked or traced back to a single activity (e.g. eIF4G cleavage or inducing NCTD), is largely unknown. Further research into these networks may allow us to fully understand how (picorna)viruses evade antiviral host responses, something that is of vital importance for *in vivo* infection and thus may provide new insights that can be used in vaccine development.

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Chapter 8

Addendum

Nederlandse samenvatting

De picornavirussen vormen een grote virusfamilie waartoe enkele bekende humane en veterinaire virussen behoren. Bekende picornavirussen zijn onder andere poliovirus, hepatitis A virus en het mond-en-klauwzeervirus. Het mond-en-klauwzeervirus was zelfs het eerste dierlijke virus – een infectieus agens dat kleiner is dan een bacterie maar wel ziekte kan overdragen – dat werd geïdentificeerd. Kort na de ontdekking van het mond-en-klauwzeervirus in 1898, werd in 1908 het poliovirus, een picornavirus dat mensen kan infecteren, geïdentificeerd. De term “virus”, afgeleid van het Latijnse woord voor vergif, werd geïntroduceerd om naar deze ziekteverwekkers te verwijzen. Het was destijds echter volstrekt onbekend wat deze ziekteverwekkers waren of hoe ze ziekte kunnen veroorzaken. Sinds hun ontdekking zijn deze twee virussen intensief bestudeerd. Onder andere dit onderzoek was een van drijfveren achter de ontwikkeling van de moleculaire virologie als zelfstandig vakgebied. Daarnaast heeft het onderzoek naar de vermeerdering van deze virussen, welke ten koste gaat van hun gastheer, bijgedragen aan onze kennis van de moleculaire processen in de cel.

Virussen zijn acellulaire parasieten die voor hun vermeerdering afhankelijk zijn van het metabolisme van hun gastheer. De vermeerdering van alle virussen, en dus ook van picornavirussen, kan worden herkend door de gastheer. De gastheer kan vervolgens enkele antivirale responsen inschakelen om de vermenigvuldiging en verspreiding van het virus tegen te gaan. Voor een succesvolle infectie is het virus genoodzaakt om deze antivirale responsen te onderdrukken of te ontduiken. Het onderzoek beschreven in dit proefschrift richt zich op de manieren waarop enkele picornavirussen de verschillende antivirale responsen onderdrukken of ontduiken. Hierbij wordt specifiek gekeken naar twee vroege antivirale reacties van een geïnfecteerde cel; de expressie van type I-interferon en het inschakelen van de cellulaire stressrespons.

De type I-interferon eiwitten (IFN- α/β) zijn centrale spelers in het aangeboren immuunsysteem. Deze pro-inflammatoire cytokinen vormen de eerste alarmsignalen en staan aan de basis van de verdere immuunrespons. VirusrePLICATIE kan worden gedetecteerd door de familie van RIG-I-like receptoren (RLRs). Dit leidt tot het inschakelen van de zogenaamde RLR-sigNaaltransductieroute, wat resulteert in de expressie van IFN- α/β . Het geproduceerde IFN- α/β kan vervolgens aan de interferonreceptor binden en zorgt voor de expressie van honderden interferon-geïnduceerde genen, waardoor deze cellen in een verdedigingsmodus, de antivirale staat, komen. Dit gebeurt op zowel de geïnfecteerde cel als op niet-geïnfecteerde omliggende cellen waardoor de ongeïnfecteerde cellen beschermd zijn. Daardoor heeft expressie van IFN- α/β een groot inperkend effect op de verspreiding van het virus. Logischerwijs hebben virussen effectieve methoden ontwikkeld

om de nadelige gevolgen van IFN- α/β expressie te voorkomen. Naast het voorkomen van IFN- α/β expressie, kunnen virussen ook voorkomen dat interferon-geïnduceerde genen tot expressie komen. Deze inteferon-geïnduceerde genen hebben veelal een antivirale werking en hun expressie wordt geïnduceerd door IFN- α/β .

Detectie van virusrePLICATIE kan ook resulteren in het inschakelen van de cellulaire stressrespons. Dit heeft tot gevolg dat eiwitproductie (de translatie) in de cel wordt onderdrukt dankzij de fosforylering van translatieinitiatiefactor 2 (eIF2), met mogelijke negatieve effecten voor de productie van nieuwe virussen. De onderdrukte translatie heeft daarnaast tot gevolg dat de overgebleven mRNA moleculen door de cel verzameld worden in zogenaamde ‘stress granules’, aggregaten van mRNA en translatie factoren. Vanuit de stress granules kan de translatie eventueel weer opgestart worden, nadat de cel de stresssituatie heeft opgelost. Van deze stress granules wordt gezegd dat ze een antivirale werking hebben, bijvoorbeeld doordat ze zouden functioneren als signaleringsplatform voor antivirale signaaltransductieroutes. Virussen hebben verschillende mechanismen ontwikkeld om de cellulaire stressrespons te onderdrukken of te ontwijken. Hierbij kan grofweg een onderscheid worden gemaakt tussen het voorkomen of ongedaan maken van de fosforylering van eIF2 om de virale translatie te bevorderen, en het tegengaan van de vorming van stress granules.

Van verschillende picornavirussen is bekend dat ze voor het onderdrukken of ontwijken van deze twee antivirale responsen gebruik maken van hun “security proteins”, virale eiwitten die niet strikt noodzakelijk zijn voor de vermenigvuldiging van het virus, maar wel een positief effect hebben op de verspreiding van het virus. In dit proefschrift beschrijven we onderzoek naar de exacte mechanismen waarmee enkele picornavirus security proteins de genoemde antivirale responsen onderdrukken of ontwijken.

In hoofdstuk 2-4, onderzochten we het mond-en-klauwzeervirus. Voor dit virus was nog weinig bekend omtrent het onderdrukken van de cellulaire stressrespons. In hoofdstuk 2 beschrijven we dat dit virus de vorming van stress granules onderdrukt via het leader eiwit. We laten zien dat een mond-en-klauwzeervirus zonder dit leader eiwit de stress granule-vorming niet langer kan onderdrukken. Het mond-en-klauwzeer leader eiwit is een protease, daarom hebben we onderzocht of leader specifieke stress granule eiwitten kan knippen. Dit bleek het geval, leader knipt de eiwitten G3BP1 en G3BP2.

Het leader protease van mond-en-klauwzeervirus is ook bekend vanwege zijn rol in de onderdrukking van type I-interferon, al is het onderliggende mechanisme niet volledig opgehelderd. De RLR-sigNaaltransductieroute wordt gereguleerd via de post-translationele modificaties fosforylering en ubiquitinering. Het is bekend dat verschillende virale

proteases door middel van deubiquitinase activiteit een negatief effect hebben op de RLR-sigtaaltransductie route. Dit mechanisme is ook voorgesteld voor het leader protease van mond-en-klauwzeervirus, al is dit voornamelijk gebaseerd op bevindingen in cellen waarin ubiquitine en leader tot expressie zijn gebracht, en dus niet op data uit geïnfecteerde cellen. In hoofdstuk 3 beschrijven wij dat leader voornamelijk deISGylase activiteit bezit en slechts heel lichte deubiquitinase activiteit heeft. ISG15 is een van de interferon-geïnduceerde genen en lijkt qua structuur erg op een di-ubiquitine keten. ISG15 kan, net als ubiquitine, covalent gebonden worden aan eiwitten, al is de rol hiervan nog onduidelijk. We verklaren de extreme specificiteit van leader voor ISG15 doormiddel van het ophelderen van de kristalstructuur van leader in complex met ISG15. Uit deze kristalstructuur blijkt dat ubiquitine een voor de interactie met leader belangrijke tryptofaan mist, welke wel aanwezig is in ISG15.

In hoofdstuk 4 onderzochten we vervolgens de rol van leader's deISGylase activiteit in het onderdrukken van type I-interferon. Het knippen van RLR-signalerings-eiwitten door leader is ook gesuggereerd om bij te dragen aan het onderdrukken van de type I-interferon productie. In dit hoofdstuk hebben we eerst systematisch onderzocht welke eiwitten in de RLR-sigtaaltransductieroute geknipt worden door leader. Met een set van mutanten hebben we onderzocht welke proteolytische activiteit (het knippen van RLR-signalerings-eiwitten of de deISGylase activiteit) bijdraagt aan het onderdrukken van de expressie van IFN- α/β . De deISGylase activiteit van leader bleek niet belangrijk te zijn, terwijl onderdrukking van type I-interferon expressie wel correleert met het knippen van RLR-signalerings-eiwitten door leader.

In hoofdstuk 5 onderzochten we de humane enterovirussen. Enterovirussen hebben twee proteasen, 2A^{pro} en 3C^{pro}. Voor beide proteasen is gesuggereerd dat ze belangrijk zijn in het onderdrukken van antivirale responsen. Hierdoor bleef het exacte mechanisme waarmee enterovirussen deze antivirale responsen onderdrukken, en het verantwoordelijke virale eiwit, onduidelijk. In dit hoofdstuk hebben we de 2A^{pro} en 3C^{pro} van representatieve enterovirussen van de verschillende humane enterovirus genera (EV-A t/m EV-D) vergeleken. Voor elk van deze proteasen hebben we onderzocht of deze de expressie van type I-interferon en de vorming van stress granules kan onderdrukken. Uit deze omvangrijke vergelijking blijkt dat voor alle geteste enterovirussen het 2A^{pro} de belangrijkste antagonist is van beide antivirale responsen. Van de stress granules is gesuggereerd dat deze een rol spelen als signaleringsplatform voor verdere antivirale responsen, bijvoorbeeld voor de inductie van type I-interferon expressie. Omdat 2A^{pro} een negatief effect heeft op zowel de expressie van IFN- α/β als op de vorming van stress granules, hebben we het gesuggereerde verband tussen deze twee antivirale responsen in meer detail onderzocht. Onze data suggereren dat stress granules een rol spelen in de RLR-sigtaaltransductieroute, maar

dat ze niet essentieel zijn voor IFN- α/β expressie. Bovendien laten wij zien dat de onderdrukking van type I-interferon expressie door enterovirus 2A^{pro} een veelvoud is van de relatief kleine bijdrage van SGs aan deze signaleringsroute, wat suggereert dat 2A^{pro} een additioneel mechanisme heeft om de RLR-sigtaaltransductieroute direct te kunnen onderdrukken.

In hoofdstuk 6 onderzochten we de kobuvirussen. Het humane kobuvirus, aichivirus, werd in de jaren '90 in Japan ontdekt, en er is nog weinig bekend over het onderdrukken van antivirale responsen door dit virus. Wij ontdekten dat de leader van dit virus, ditmaal geen protease, een uniek mechanisme heeft om de cellulaire stressrespons te onderdrukken. Zoals eerder beschreven kunnen virussen de cellulaire stressrespons onderdrukken op grofweg twee manieren; het tegengaan of omzeilen van de sigtaaltransductie route waardoor er geen sprake is van p-eIF2, of het tegengaan van de vorming van stress granules. In dit hoofdstuk beschrijven we dat het leader eiwit van aichivirus een directe interactie aangaat met het eIF2B complex, waardoor translatie kan plaatsvinden in de aanwezigheid van gefosforyleerd eIF2. In een niet-geïnfecteerde cel, gaat eIF2 een interactie aan met het eIF2B complex om geladen te worden met een nieuw GTP en zo gebruikt te kunnen worden in een volgende ronde van translatie. Ten tijde van stress kan de cel eIF2 fosforyleren. Het gevormde p-eIF2 heeft een inhiberend effect op het eIF2B complex, waardoor het niet-gefosforyleerde eIF2 niet langer wordt gerecycled en de translatie tot stilstand komt. In aanwezigheid van aichivirus leader zien wij dat translatie ongehinderd is, ondanks de aanwezigheid van p-eIF2. Eenzelfde mechanisme is slechts een keer eerder geïdentificeerd, het accessory protein 10 van beluga whale coronavirus bindt ook aan eIF2B om translatie in aanwezigheid van p-eIF2 te faciliteren. Vervolgens hebben we onderzocht of dit mechanisme geconserveerd is in de andere kobuvirussen. Dit bleek enkel het geval voor het leader eiwit van het nauwverwante muizenkobuvirus, wat suggereert dat de onderdrukking van de cellulaire stressrespons door leader een recente evolutionaire ontwikkeling is, en leader hoogstwaarschijnlijk een andere primaire functie heeft.

Dankwoord

Welcome dear readers, you have made it to the most sought-after section of any thesis, the acknowledgments. In your hands is the result of 5 years of hard work. I want to invite you to also have a quick look at the other chapters of this thesis before putting it on your bookshelf and returning to the lab. I would like to thank everyone who contributed to my scientific development, in any way, shape or form. Completing a PhD project is a truly life-changing event.

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About the author

Linda Jessica Visser was born on May 26th 1991 in Apeldoorn, the Netherlands. In 2009 she graduated from bilingual pre-university education (T-VWO) at the Koninklijke Scholengemeenschap in Apeldoorn. That same year, she started a bachelor degree in Biomedical Sciences at Utrecht University, obtaining her degree in 2012. Linda continued her education via the master program Infection & Immunity at Utrecht University. As a part of this program she conducted an internship at the UMC Utrecht medical center under the supervision of dr. M.L. van de Weijer, dr. R.J. Lebbink and prof. E.J.H.J. Wiertz. Here, she studied how the CMV US2 protein interferes with antigen presentation. A second internship under supervision of prof. G. van den Bogaart was conducted at the Radboud Institute for Molecular Life Sciences. Here, Linda investigated the molecular details of the immunological synapse in dendritic cells. During this master program, Linda got the opportunity to apply for PhD project funding via the NWO graduate program. The research proposal written by her, dr. M.A. Langereis and prof. F.J.M. van Kuppeveld acquired funding, and in 2014 Linda started as a PhD candidate at the Faculty of Veterinary Medicine at Utrecht University. She studied the strategies employed by picornavirus security proteins to evade or counteract innate antiviral responses. The results on this research are described in this thesis.

List of publications

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Visser LJ, Aloise C, Swatek KN, Medina GN, Olek KM, Rabouw HH, de Groot RJ, Langereis MA, de Los Santos T, Komander D, Skern T, van Kuppeveld FJM. 2020. *Dissecting distinct proteolytic activities of FMDV Lpro implicates cleavage and degradation of RLR signaling proteins, not its deISGylase/DUB activity, in type I interferon suppression*. PLoS Pathog.

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