Enteroviral proteases: structure, host interactions and pathogenicity

Olli H. Laitinen1, Emma Svedin2+, Sebastian Kapell2+, Anssi Nurminen1+, Vesa P. Hytönen1 and Malin Flodström-Tullberg1,2*

1BioMediTech, Finland and Fimlab Laboratories, University of Tampere, Tampere, Finland
2The Center for Infectious Medicine, Department of Medicine HS, Karolinska Institutet, Stockholm, Sweden

SUMMARY

Enteroviruses are common human pathogens, and infections are particularly frequent in children. Severe infections can lead to a variety of diseases, including poliomyelitis, aseptic meningitis, myocarditis and neonatal sepsis. Enterovirus infections have also been implicated in asthmatic exacerbations and type 1 diabetes. The large disease spectrum of the closely related enteroviruses may be partially, but not fully, explained by differences in tissue tropism. The molecular mechanisms by which enteroviruses cause disease are poorly understood, but there is increasing evidence that the two enteroviral proteases, 2Apro and 3Cpro, are important mediators of pathology. These proteases perform the post-translational proteolytic processing of the viral polyprotein, but they also cleave several host-cell proteins in order to promote the production of new virus particles, as well as to evade the cellular antiviral immune responses. Enterovirus-associated processing of cellular proteins may also contribute to pathology, as elegantly demonstrated by the 2Apro-mediated cleavage of dystrophin in cardiomyocytes contributing to Coxsackievirus-induced cardiomyopathy. It is likely that improved tools to identify targets for these proteases will reveal additional host protein substrates that can be linked to specific enterovirus-associated diseases. Here, we discuss the function of the enteroviral proteases in the virus replication cycle and review the current knowledge regarding how these proteases modulate the infected cell in order to favour virus replication, including ways to avoid detection by the immune system. We also highlight new possibilities for the identification of protease-specific cellular targets and thereby a way to discover novel mechanisms contributing to disease. Copyright © 2016 John Wiley & Sons, Ltd.

INTRODUCTION

Enterovirus infections are among the most common types of virus infections in humans. The majority of infections are subclinical, but occasionally, they cause diseases such as the common cold, hand-foot-and-mouth disease (HFMD), myocarditis meningitis, otitis media, neonatal sepsis, pancreatitis, poliomyelitis and sinusitis [1,2]. In addition, enterovirus infections have been associated with inflammatory diseases, such as type 1 diabetes, asthma and allergies [1,3]. Our understanding of the complex processes leading to these different disorders is limited, and a better knowledge of how these viruses interact with the host is essential for the discovery of

Abbreviations used

2Apro, enteroviral protease 2A; 3Cpro, enteroviral protease 3C; HFMD, hand-foot-and-mouth disease; PRR, pattern recognition receptor; VP, viral capsid protein; IgSF, immunoglobulin superfamily; ICAM-1, intracellular adhesion molecule; CAR, coxsackievirus–adenovirus receptor; PVR, poliovirus receptor; DAF, decay accelerating factor; LDLR, low-density lipoprotein receptor; PTB, polypyrimidine tract-binding protein; 3DP, enteroviral RNA-dependent RNA polymerase; 3B, uridylylated-VPg; EV, extracellular vesicle; EV71, enterovirus 71; EV68, enterovirus 68; CVB, coxsackievirus; SRF, serum response factor; miRNA, micro RNA; HRV, human rhinovirus; eIF4G, eukaryotic translation initiation factor 4 gamma 1; IRES, internal ribosome entry site; CRB, CAMP response element-binding protein; Oct-1, octamer binding transcription factor 1; NLS, nuclear localization signal; NPC, nuclear pore complex; SRp20, cellular splicing factor; PCBP, cellular RNA-binding protein poly(rC)-binding protein; dsRNA, double stranded RNA; IFIH1, interferon induced with helicase C domain 1; TLR, toll-like receptor; RIG-I, retinoic acid-inducible gene I; MAVS, mitochondrial antiviral-signalling protein 1; SCARB2, scavenger receptor B2; TRIF, TIR-domain-containing adapter-inducing interferon-B; ISG, interferon-stimulated gene; G3BP1, Ras GTPase-activating protein-binding protein.

Copyright © 2016 John Wiley & Sons, Ltd.
disease-causing mechanisms and the identification of targets for the development of therapeutic measures. All enteroviruses encode two proteases, 2A (2A<sup>PP</sup>) and 3C (3C<sup>PP</sup>), which are essential for the cleavage of the viral polyprotein into structural- and non-structural proteins. These proteases can also cleave host-cell proteins, and cellular targets already identified include transcription factors, proteins controlling nuclear import/export, mitochondria-associated proteins, pattern recognition receptors (PRRs) and other proteins, many of which are involved in the activation of the host immune response [4–13]. The cleavage of host-cell proteins may contribute to pathology [14–16], and a better insight into the target specificities of the enteroviral proteases, coupled with information on how protein cleavages affect the biological functions of the cell, is likely to reveal novel disease mechanisms as well as identify ways to treat and prevent enterovirus-mediated diseases.

CLASSIFICATION AND STRUCTURE OF ENTEROVIRUSES

The molecular characteristics, such as the nature of replication, morphology and physiochemical properties of the virion define the genus <i>Enterovirus</i>. The genus belongs to the family of Picornaviridae, under the order of Picornavirales, and the genus is divided into twelve species: <i>Enterovirus</i> A–H, J and <i>Rhinovirus</i> A–C [17].

The enterovirus virion contains a single positive strand RNA genome with a length of around 7.5 kb. The genome is densely packed into an icosahedral capsid, which is composed of 60 copies of the viral polyprotein into structural- and non-structural proteins. These proteases can also cleave host-cell proteins, and cellular targets already identified include transcription factors, proteins controlling nuclear import/export, mitochondria-associated proteins, pattern recognition receptors (PRRs) and other proteins, many of which are involved in the activation of the host immune response [4–13]. The cleavage of host-cell proteins may contribute to pathology [14–16], and a better insight into the target specificities of the enteroviral proteases, coupled with information on how protein cleavages affect the biological functions of the cell, is likely to reveal novel disease mechanisms as well as identify ways to treat and prevent enterovirus-mediated diseases.

THE ENTEROVIRUS LIFE CYCLE

Enterovirus receptors and virus entry

Enteroviruses use several types of cell-surface molecules for binding and initiating their entry into cells (Figure 1). The majority of the known enterovirus receptors belong to the immunoglobulin superfamaly (IgSF) [18], and more specifically, the type I transmembrane glycoproteins. They include the intracellular adhesion molecule (ICAM-1) [19], the coxsackievirus–adenovirus receptor (CAR) [20] and the poliovirus receptor (PVR) [21]. Non-IgSF type receptors include decay accelerating factor (DAF), the low-density lipoprotein receptor (LDL-R), scavenger receptor B2 (SCARB2) and integrins [22–26].

The tissue and cell distribution of virus receptors is an important determinant for virus tropism. Polioviruses primarily infect human gastrointestinal lymphoid tissues, such as tonsils and Peyer’s patches expressing the PVR [27,28]. If the virus spreads to the circulation and, thereafter, to the central nervous system, neuronal cells expressing PVR can become infected, resulting in muscle weakness and paralysis.

Through their attachment to the cell-surface receptors, enteroviruses gain access into the cell via endocytotic pathways. Routes of entry depend on the species of the virus and the cell type. The caveolae- [29] and the clathrin-dependent pathways [30], as well as other internalization routes [31], have been described as possible entry mechanisms. The presence of a receptor on the cell surface is, however, not the only determinant for cellular permissiveness. The virus may enter the cell but fail to replicate if, for example, there is a lack of endogenous cellular proteins required for viral propagation. An example of such an endogenous protein is the polypyrimidine tract-binding protein (PTB) [32]. Alternatively, the receptor-expressing cell can enter an antiviral state and thereby, may not be permissive to infection (reviewed in [33]). Therefore, the dependence on various cellular factors makes host susceptibility and permissiveness to infection a multifaceted and complex phenomenon.

Enterovirus translation and replication

After endocytosis, the virus particle undergoes structural changes, resulting in the uncoating of the viral genome and engagement of the capsid proteins with the endosomal membrane, presumably via the VP1 N-terminus. This allows the delivery of viral RNA with a 5’-linked VPg protein [34] and a 3’-polyadenylated tract [35] into the cytosol, where it is translated by the host ribosomes into the viral polyprotein.

The polyprotein encoded by a single open reading frame is divided into three regions, P1–P3 (Figure 1). The P1 region contains four structural
proteins (VP1–VP4), whereas the P2 and P3 regions together contain seven non-structural proteins (2A–2C and 3A–3D), which are required in the different stages of the viruses’ replication cycle. The proteolytic processing of the polyprotein into separate proteins is already initiated during translation by the viral proteases 2Apro and 3Cpro. The P1 region, encoding the structural proteins of the capsid, is the first one to be translated, followed by the P2 region, which contains three non-structural proteins (2A, 2B and 2C). During the translation of the P2 region, as 2Apro is translated first, 2Apro makes an in cis cleavage, separating itself and the P2 region from the P1 region before the full polyprotein has been translated. Translation continues through the P3 region, and this region includes the second protease, 3Cpro, which is responsible for eight out of the 10 cleavages of the viral polyprotein. The cleavage carried out by the two proteases give rise to all of the non-structural proteins, with several precursor proteins, and three structural proteins: VP1, VP3 and VP0. VP0 is further cleaved into VP2 and VP4 by an unknown mechanism [39], which may entail an RNA-mediated autocatalytic reaction during the encapsidation process [40].

Viral replication takes place in the proximity of membranous vesicles, derived partly from the endoplasmic reticulum [41]. The positive strand RNA is transcribed by the virally encoded polymerase 3Dpol into a complementary negative strand RNA. The RNA synthesis is primed by uridylylated-VPg (3B), which is associated with the replication complex and recruited to the 3’ end of the negative strand viral genome to initiate RNA synthesis [42]. The negative strand RNA then serves as a template for the transcription of the positive strand RNA genome. Multiple positive-strand RNAs can be synthesized from a single negative-strand template, making

---

Figure 1. Proposed model of the enterovirus replication cycle. (1) Entry. After attachment to host-cell surface receptors virus is internalized and uncoated, leading to the release of viral RNA into the cytoplasm. (2) Translation. Viral polyprotein is translated and then processed by the 2Apro and 3Cpro proteases. Host-cell translation is also perturbed as a component of the translation machinery (eIF4G) cleaved by 2Apro. (3) Immune evasion. Host-cell immune response is blunted by proteolysis mediated by viral proteases 2Apro and 3Cpro as intracellular receptors (MDA5/RIG-1), and proteins relaying innate signalling (IPS-1) are targeted, blocking the production of interferons and cytokines. (4) Replication. Viral proteins, in orchestration with host-cell factors, replicate the viral RNA at membrane-associated replication sites. (5) Release. Enteroviral positive-stranded RNA genomes are encapsidated by the viral structural proteins, and the new viral progeny are released either by cell lysis or in extracellular vesicles.
positive-sense RNA abundant and directly available for translation, synthesis of additional negative-sense RNA and encapsidation [43] (Figure 1).

Encapsidation and virus release
The accumulation of newly synthesized viral RNA and structural proteins leads to packaging of the viral genome into the capsids, thus forming new viral progeny [44]. Surprisingly, very little is known about the encapsidation process, but some studies have indicated that the process of virus assembly is coupled to RNA synthesis [45] on the surface of cytoplasmic membranes [46].

The classical view of enterovirus release is that it occurs by cell lysis. Intriguingly, new observations challenge this model as virus-containing extracellular vesicles shed by the host cells could potentially disseminate the infection [47,48]. Persistent enterovirus infections without evident cytopathic effect in tissues and cell models have also been reported [49–51], supporting this recently described nonlytic model of virus release.

ENTEROVIRUS-MEDIATED DISEASES
The most well-known enteroviral disease is poliomyelitis, which is caused by three different poliovirus serotypes. Poliomyelitis has been virtually eradicated in developed countries, but recently, two other enteroviruses, enterovirus 71 (EV71) and enterovirus 68 (EV68), have been demonstrated to cause an acute flaccid paralysis resembling poliomyelitis [52–55]. Moreover, EV71 and coxsackievirus A6, A10 and A16 can cause HFMD [56]. Other enteroviruses, coxsackieviruses (CVBs) in particular, have been associated with acute myocarditis and the later development of dilated cardiomyopathy [14,15,57,58].

Diseases related to enterovirus infections may result either from an acute infection or only appear after the acute phase is over. This indicates that there may be different mechanisms contributing to tissue pathology. Acute infections are typically associated with local inflammation (e.g. the common cold, otitis, pancreatitis and hepatitis) and are cleared relatively rapidly by the immune system. In contrast, conditions like dilated cardiomyopathy and post-polio syndrome are more likely to result from infections that have not been completely cleared and have entered a persistent infection phase.

Although poliomyelitis caused by poliovirus is the most studied enterovirus-associated disease, surprisingly little is known about the disease mechanisms [59]. Even less is known on how most other enteroviruses cause disease (e.g. EV71 and EV68). An exception, however, is CVB-induced myocarditis and the subsequent development of chronic dilated myopathy, the latter a severe condition that usually leads to heart failure [15,58]. During the acute phase of the infection, the virus-encoded protease 2Apro cleaves the cellular protein dystrophin, which leads to sarcolemmal disruption and reduction in myocyte contractility [14,57]. In their recent publication, Matthew *et al*. postulated a more detailed molecular mechanism for the damage caused by the infection, namely that the C-terminal 2Apro cleavage product is retained in the sarcoglycan complex. This in turn decouples actin from the sarcolemma and subsequently prevents the recovery of the full-length dystrophin at the sarcolemmal membrane [16].

A further contribution to impaired cardiac function is the 2Apro-mediated cleavage of the transcription factor serum response factor (SRF) [60]. SRF is normally highly expressed in heart muscle cells and contributes to the regulation and expression of heart tissue-specific genes, including contractile and regulatory proteins as well as miRNAs controlling specific heart cell functions [61]. The 2Apro breaks the transactivation domain of SRF and thereby diminishes the expression of genes regulated by this transcription factor [60].

Coxsackieviruses have been shown to cause persistent infection of the heart both in animal models [62,63] and humans [64]. Characteristic of other persistent CVB infections, they also contain deletions of varying size in their 5′ end [64]. The persistent infection may lead to a chronic immune response and also possibly autoimmune responses as exemplified by antibody responses to cardiac antigens such as cardiac myosin and troponin I [15]. The chronic inflammation is likely to contribute further to cardiac dysfunction.

THE ENTEROVIRUS-ENCODED PROTEASES 2Apro AND 3Cpro

Structural features of enterovirus proteases
The enterovirus proteases 2Apro and 3Cpro are multifunctional cysteine proteases, belonging to the chymotrypsin-related endopeptidase protease family [65] (MEROPS 2Apro: C03.020 and 3Cpro: C03.021).
When comparing 2A<sub>pro</sub> to 3C<sub>pro</sub>, a primary sequence alignment of the consensus sequences shows only ~20% identity, even though the two proteases have strikingly similar tertiary structures (Figure 2). Among the different species of enteroviruses, the proteases share approximately 50–75% sequence identity, the rhinoviruses being the most divergent group with around 35–55% identity with the other species (Figure 3). The amino acid residues of the catalytic triad are fully conserved throughout the <i>Enterovirus</i> genus. In addition, the amino acid residues surrounding the catalytic residues are more conserved when compared to the rest of the protein, which is indicative of similarities in the mechanisms involving sequence specificity and cleavage among the enteroviral proteases.

The tertiary structures of both of the proteases are composed of two separate domains. In the case of 2A<sub>pro</sub>, the two domains include a six-stranded antiparallel β-sheet barrel and a β-sheet pile packed on its side (Figure 2). The tertiary structure of 3C<sub>pro</sub>
is a combination of two twisted β-barrels, which are packed perpendicular to each other. In both proteases, these two domains participate in the formation and positioning of the catalytic triad. The catalytic triad is composed of histidine, aspartic acid and cysteine in the case of 2Apro, and histidine, glutamic acid and cysteine in the case of 3Cpro. The cysteine in the catalytic triad acts as a nucleophile in the proteolytic reaction in both proteases. Characteristic for both proteases are also the conserved ion-binding motifs that are located on the opposite side from the catalytically active site. For 2Apro, a zinc ion is located in one end of the barrel, bound by three cysteines and one histidine residue. For 3Cpro, a chlorine ion is bound to an Asp-Ile-Arg stretch residing in the loop connecting the two barrels.

Both monomeric and dimeric quaternary structure forms have been reported for 2Apro. Liebig et al. found that HRV2 2Apro showed a dimeric state in gel filtration analysis, while CVB4 2Apro was found to be monomeric [67]. In another study, 2Apro from HRV14 was found to be monomeric by gel filtration analysis [68]. In a study by Cai et al., EV71 2Apro was found to form a disulphide-linked dimer with a negligible monomer–monomer interface in crystal structure, but the oligomeric state in solution could not be shown [69]. Mu et al. crystallized EV71 2Apro and found a monomer in the asymmetric unit [70]. In another recent study of CVA16 2Apro, both dimeric and hexameric quaternary assemblies in the solution and in crystal were reported [71]. The hexameric form was found to dissociate to dimers with an addition of DTT, which could indicate that the hexamer is not present in the reducing intracellular environment. Both dimers and hexamers, separated by size exclusion chromatography, exhibited equally efficient proteolytic activity.

It is most likely that the quaternary structure of 3Cpro is monomeric because it lacks a third domain, whose importance has been shown for dimerization in related coronavirus proteases [72,73]. This is in contrast to what has been observed when solving the crystal structure, in which 3Cpro proteases assembled as dimers. For example, 3Cpro from EV68 and EV93 showed a dimeric assembly in crystal structures. On the contrary, they were found to be monomeric in gel filtration and DLS experiments [74,75]. Therefore, the dimers observed in crystals are not likely to represent the biologically relevant forms.

**Sequence specificity of enteroviral proteases**

The sequence specificity, and specifically the sequences that the 2Apro and 3Cpro proteases are able to cleave (or not), has not been established or studied comprehensively. To date, the amino acid residues P4, P2, P1, P1′ and P2′ are recognized as being important determinants for the sequence specificity of enteroviral proteases (Figure 4) [65,76]. For the substrate recognition of 2Apro, the most important residue is P1′, which is exclusively a glycine. Following P1′ in order of importance are P2, occupied mainly by threonine and asparagine; P2′, occupied by proline, alanine and phenylalanine; and P4, occupied most frequently by leucine or threonine. For 3Cpro, the residues P1 and P1′ show the least amount of variance in the substrate sequence. The preferred residues for these positions are glutamine or glutamate for P1, and glycine, asparagine or serine for P1′. In addition, the most common residue is alanine in position P4 and proline in position P2′. The most obvious feature for determining the substrate specificity of both 2Apro and 3Cpro is the strong conservation of the glycine residue in position P1′ [76], and the present understanding of which residues are important in the other positions may be revised as new information becomes available (refer to the ‘Methods to Identify New Cellular Substrates for Enteroviral Proteases’ and ‘Cleavage Predictions Using in Silico Analysis Techniques, Bioinformatics’ sections in the succeeding texts).

**Protease inhibitors as antiviral compounds**

As the protease-dependent processing of the enteroviral polyprotein is indispensable for virus replication, the viral proteases have been recognized as potential targets for antiviral intervention [79,80]. Of the two proteases, 3Cpro in particular, has been considered a compelling target, as the polyprotein has several cleavage sites specific for the protease. Many of the inhibitors that have been developed and studied are small molecule peptide mimetics that target the active site of the proteases, but other small molecular compounds have also been described [81]. Structural conservation and the commonly shared proteolytic mechanism seen between different viral proteases make it possible to develop inhibitors that have an antiviral activity towards many species in the Enterovirus genus and furthermore, occasional activity towards more
distantly related viruses. Such inhibitor candidates include pyrazole compounds that target 3C\textsuperscript{pro} from different enteroviruses as well as coronavirus protease homologues of 3C\textsuperscript{pro} [79], microcyclic inhibitors against enterovirus 3C\textsuperscript{pro} and noro- and SARS-coronavirus 3C\textsuperscript{pro} homologues [82]. Additionally, a lycorine derivative, 1-acetyllycorine, has been shown to inhibit EV71 2A\textsuperscript{pro} by stabilizing a special conformation of its zinc finger motive. Similarly, it can furthermore act on the homologous zinc finger of Hepatitis C virus NS3 protease [81]. The rhinovirus 3C\textsuperscript{pro} inhibitor rupintrivir [83] is also active against noroviruses [84].

To date, of all the compounds studied, only rupintrivir and its analogue AG7404 (or compound 1) [85] have progressed to clinical trials [85–87]. Their development as therapeutics for rhinovirus infection has since stalled, possibly a result of their limited activity in clinical trials [88,89]. Recently, rupintrivir has, however, gained renewed attention as it proved to be effective against EV71, CAV16 and EV68 [90–93]. These interesting and optimistic results put renewed focus on the development of antivirals that target viral proteases, and it is possible that one or several novel drug candidates may show efficacy in clinical trials and reach the market in the coming years.

Interactions between 2A\textsuperscript{pro} and 3C\textsuperscript{pro} with host-cell transcription and translation machinery

As mentioned in the preceding texts, the enterovirus proteases fulfil several other functions in addition to cleaving the viral polyprotein into mature viral proteins. For example, they cleave cellular proteins in order to favour viral propagation over cellular protein production. The protease 2A\textsuperscript{pro} interferes with and shuts down host-cell protein synthesis through cleavage of eukaryotic translation initiation factor 4 gamma 1 (eIF4G) [8], an essential component of the cap-dependent RNA translation machinery. As enteroviruses are lacking a 7-methylguanosine cap, the cleavage of eIF4G will...
not affect viral protein synthesis. Instead, the enteroviruses use a highly ordered secondary structure in the 5′ end of the viral RNA called the internal ribosome entry site (IRES) to achieve the initiation of translation [94,95].

Host-cell gene transcription is also affected by enterovirus infection. During infection, the 3CD precursor protein enters the nucleus and inhibits the transcription of cellular proteins by cleavage of the TATA box, cAMP response element-binding protein, octamer binding transcription factor 1 (Oct-1) and transcriptional activating factor p53 [9,96–98]. Although the polymerase in 3CD contains a nuclear localization signal (NLS) [99], a recent study showed that 2Apro-mediated proteolysis is required for the nuclear translocation of 3CD [100].

In addition to a direct cleavage of cellular proteins (Tables 1 and 2; for more complete list of published substrates, refer to Tables S1 and S2), the proteases can also indirectly affect cellular proteins to further promote viral replication. For example, 2Apro targets several nuclear pore complex (NPC) proteins like Nup62, -98 and -153 [114,115].

Table 1. Examples of published enteroviral 2A substrates

| Target protein                                      | Virus       | Refseq/ UniProtKB AC       | Gene         | Cleavage site (sequence) |
|-----------------------------------------------------|-------------|-----------------------------|--------------|--------------------------|
| Dystrophin                                          | CVB3        | NP_000100/P11532            | DMD          | PGLTTI2434-GASP          |
| eIF4GI                                              | CVB4 Polio | NP_886553/Q04637            | EIF4G1       | TTLSTR681-GPRR           |
| Melanoma differentiation-associated protein 5(MDA5) | EV71        | NP_071451/Q9BYX4            | IFIH1        | RTVATS53-GNMQa           |
| Interferon (α, β and ω) receptor 1                  | EV71        | NP_000620/P17181            | IFNAR1       | RSDESV56-GNVTa           |
| Nucleoporin 62                                       | Polio RV16  | NP_001180286/P37198         | NUP62        | PATQTT72-GFPAa           |
| Nucleoporin 98                                       | Polio HRV2  | NP_005378/P52948            | NUP98        | ATITST217-GPSLa          |
|                                                      | HRV16       |                             |              | TVPVTAA246-GAPTa         |
|                                                      |             |                             |              | EHLNTS161-GAPAa          |
|                                                      |             |                             |              | VGSTLF174-GNNNK          |
|                                                      |             |                             |              | KALQTT1352-GTAKa         |
| Nucleoporin 153                                      | Polio       | NP_001265138/P49790         | NUP153       | SCTVT781-GTGLa           |
| Serum response factor                                | CVB3        | NP_003122/P11831            | SRF          | QTTSST1260-GTAVa         |
|                                                      |             |                             |              | NNTTTS1287-GFGEa         |
|                                                      |             |                             |              | TVLKST326-GSGP           |

Equal to UniprotKB sequence P52948 amino acid G569.

*aPredicted, unconfirmed cleavage site (Nurminen et al. Manuscript in preparation).
disrupts the NPC and results in the rearrangement of nuclear proteins into the cytoplasm, where viral replication occurs. An example of a protein that is redistributed in this process is cellular splicing factor (SRp20), which binds the cellular RNA-binding protein poly(rC)-binding protein (PCBP) and recruits ribosomes to the replicating viral RNA to promote IRES-dependent initiation of the translation [114,116]. Thus, the relocation of cellular transcription factors is utilized to modulate both viral translation and at a later stage, the generation of a new viral RNA genome [117–119].

Table 1. Examples of published enteroviral 2A substrates

| Target protein | Substrate’s cellular localization/function | Consequence(s) of proteolytic cleavage | Ref. |
|----------------|------------------------------------------|--------------------------------------|------|
| Dystrophin     | Cytoplasmic/Connects the cytoskeleton of a muscle fibre to the surrounding extracellular matrix | Sarcolemmal disruption leading to myocarditis and cardiomyopathy | [14] |
| eIF4GI         | Cytoplasmic/Translation initiation        | Decline of host-cell protein synthesis | e.g. [101,102] |
| Melanoma differenation-associated protein 5 (MDA5) | Nuclear, cytosolic/cellular processes involving translation initiation, nuclear and mitochondrial splicing and ribosome and spliceosome assembly | Inhibition of type I Interferon response | [4,10] |
| Interferon (α, β and ω) receptor 1 | Cell membrane/mediates type I interferon signalling | Antagonizes type I interferon signalling | — |
| Nucleoporin 56 | —                                        | —                                    | [7] |
| Nucleoporin 98 | Nuclear membrane/traffic of biological molecules between the nucleus and the cytoplasm | Prevent mRNA trafficking from nucleus to cytoplasm. Relocation of cellular proteins and inhibition of nuclear import/export. | [103–105] |
| Nucleoporin 153 | —                                        | —                                    | [103] |
| Serum response factor | Nucleus/Cardiac-enriched transcription factor | Impaired cardiac function by downregulation of cardiac-specific contractile and regulatory genes | [60] |

The role for 2A<sup>Pro</sup> and 3C<sup>Pro</sup> in immune evasion
Infected cells have several intracellular receptors that recognize different types of viruses. The enteroviruses form a dsRNA structure during replication, and the main known receptors responsible for sensing enteroviruses are interferon induced with helicase C domain 1 (IFIH1) located in the cytoplasm and toll-like receptor 3 (TLR3) in the endosomes. IFIH1 and the closely related PRR retinoic acid-inducible gene I (RIG-I) signal via a common adaptor protein called mitochondrial...
antiviral-signalling protein 1 (MAVS), while TLR3 signals via TIR-domain-containing adapter-inducing interferon-B (TRIF). Signalling via MAVS and TRIF results in the phosphorylation of several transcription factors such as IRF3, IRF7 and NFkB, which then migrate into the nucleus and induce the expression of type I and III interferons (IFNs) as well as other inflammatory cytokines (Figure 1) [30,108,120,121]. Secreted IFNs act in an autocrine or paracrine manner to trigger the cells into entering an antiviral state by the induced expression of interferon-stimulated genes (ISGs) [120,122].

In addition to manipulating cellular proteins to favour viral replication, enteroviruses also utilize

| Target protein                                      | Virus   | Refseq/UniProtKB AC | Gene   | Cleavage site (sequence) |
|-----------------------------------------------------|---------|---------------------|--------|--------------------------|
| Cleavage stimulation factor (Cst-64)                | EV71    | NP_001293138/P33240 | CSTF2  | LMQASM250-QGGV one or more of glycines: 483, 496, 505, 510 and 515 YIAITQ185-GGA1 |
| CRE-binding protein/cyclic AMP-responsive element-binding protein 1 | Polio   | NP_004370/P16220    | CREB1  |                          |
| Interferon regulatory factor 7, IRF7                | EV71    | NP_001563/Q92985    | IRF7   | LLQAVQQ189-SCLA           |
| Mitochondrial antiviral signalling protein (MAVS)   | CVB3    | NP_065797/Q7Z434    | MAVS   | PVQETQ0148-APES           |
| Nucleoporin 62                                      | RV14    | NP_714941/P37198    | NUP62  | Many potential cleavage sites |
| Nucleoporin 153                                     | RV14 RV16 | NP_001265138/P49790 | NUP153 | Many potential cleavage sites KLGFTQ329-GDVG |
| Octamer binding transcription factor                | Polio   | NP_002688/P14859    | POLU1F1 (OCT1) | DDX58 KMIQTR728-GRGR |
| Probable ATP-dependent RNA helicase, RIG-I          | Polio echo1 RV16 | NP_055129/O95786 | DDX58 | KMIQTR728-GRGR |
| p65-RelA, transcription initiation factor TFIID subunit 4B | Polio   | NP_001230913/Q04206 | RELA   | QQLLNQ480-GIPV           |
| TATA-binding protein (TBP)                          | Polio   | NP_003185/P20226    | TBP    | GLASQ418-GAMT             |
| TRIF, toll-like receptor adaptor molecule 1         | CVB3    | NP_067681/Q86XR7    | TICAMI (TRIF) | TPFALQ190-TINA |

*Predicted, unconfirmed cleavage site (Nurminen et al. Manuscript in preparation).
the proteases to escape recognition by the immune system. It has been shown that both 2A\textsuperscript{Pro} and 3C\textsuperscript{Pro} cleave several proteins within the viral recognition pathway, thus inhibiting the induction of IFNs. For example, viral sensors like IFIH1 and RIG-I are targets of the proteases [4,10–12]. In addition, TRIF and MAVS, both adaptor proteins for the two major RNA sensing pathways TLR3 and IFIH1/RIG-I, are cleaved by 3C\textsuperscript{Pro} and/or 2A\textsuperscript{Pro} [4,5,13,108], and downstream proteins like the transcription factor IRF7 can be targeted as well [5]. It has also been shown that EV71 2A\textsuperscript{Pro} acts directly on the interferon receptor 1, reducing its expression and thereby impairing the efficacy of IFN as a treatment against infection [7].

Table 2. Examples of published enteroviral 3C substrates

| Target protein                                                                 | Substrate’s cellular localization/function                                                                 | Consequence(s) of proteolytic cleavage                                                                 | Ref.   |
|--------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|--------|
| Cleavage stimulation factor (Cst-64)                                           | Nucleus/Recognizes the second polyadenylation sequence element on pre-mRNA                                | Impairs cellular 3‘-end pre-mRNA processing and polyadenylation.                                       | [106]  |
| CRE-binding protein/cyclic AMP-responsive element-binding protein 1            | CRE-binding protein/cyclic AMP-responsive element-binding protein                                         | Inhibition of CREB-activated transcription in host cells                                               | [107]  |
| Interferon regulatory factor 7, IRF7                                           | Nucleus/Transcription factor                                                                              | Inhibits IFN gene expression                                                                         | [5]    |
| Mitochondrial antiviral signalling protein (MAVS)                               | Mitochondrial antiviral-signalling protein                                                                | Inhibition of types I and III interferon response — MAVS release from mitochondria, and morphological and functional changes of mitochondria | [13,108] |
| Nucleoporin 62                                                                 | Nuclear membrane/traffic of biological molecules between the nucleus and the cytoplasm                  | Relocation of cellular proteins and inhibition of nuclear import                                      | [109]  |
| Nucleoporin 153                                                                | —                                                                                                         | Prevent mRNA trafficking from nucleus to cytoplasm                                                   | [104,109,110] |
| Octamer binding transcription factor                                           | Nucleus/Transcription factor                                                                              | Lost inhibition of transcriptional activation by the SV40 B enhancer                                  | [79,111] |
| Probable ATP-dependent RNA helicase, RIG-I                                     | Cytoplasmic/Putative RNA helicase involved in viral RNA binding                                           | Attenuate virus recognition and the innate immune response                                           | [11]   |
| p65-RelA, transcription initiation factor TFIID subunit 4B                     | Nuclear factor of kappa light polypeptide gene enhancer in B-cells NF-kB complex                          | Suppression of NF-kB response                                                                       | [112]  |
| TATA-binding protein (TBP) TRIF, toll-like receptor adaptor molecule 1        | Nucleus/Transcription factor                                                                              | May inhibit RNA polymerase II                                                                        | [9,113] |
|                                                                                | Cytoplasm: signalosome                                                                                    | May suppress the types I and III IFN signalling and apoptosis                                        | [13,108] |

---

Table 2. Examples of published enteroviral 3C substrates

| Target protein                                                                 | Substrate’s cellular localization/function                                                                 | Consequence(s) of proteolytic cleavage                                                                 | Ref.   |
|--------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|--------|
| Cleavage stimulation factor (Cst-64)                                           | Nucleus/Recognizes the second polyadenylation sequence element on pre-mRNA                                | Impairs cellular 3‘-end pre-mRNA processing and polyadenylation.                                       | [106]  |
| CRE-binding protein/cyclic AMP-responsive element-binding protein 1            | CRE-binding protein/cyclic AMP-responsive element-binding protein                                         | Inhibition of CREB-activated transcription in host cells                                               | [107]  |
| Interferon regulatory factor 7, IRF7                                           | Nucleus/Transcription factor                                                                              | Inhibits IFN gene expression                                                                         | [5]    |
| Mitochondrial antiviral signalling protein (MAVS)                               | Mitochondrial antiviral-signalling protein                                                                | Inhibition of types I and III interferon response — MAVS release from mitochondria, and morphological and functional changes of mitochondria | [13,108] |
| Nucleoporin 62                                                                 | Nuclear membrane/traffic of biological molecules between the nucleus and the cytoplasm                  | Relocation of cellular proteins and inhibition of nuclear import                                      | [109]  |
| Nucleoporin 153                                                                | —                                                                                                         | Prevent mRNA trafficking from nucleus to cytoplasm                                                   | [104,109,110] |
| Octamer binding transcription factor                                           | Nucleus/Transcription factor                                                                              | Lost inhibition of transcriptional activation by the SV40 B enhancer                                  | [79,111] |
| Probable ATP-dependent RNA helicase, RIG-I                                     | Cytoplasmic/Putative RNA helicase involved in viral RNA binding                                           | Attenuate virus recognition and the innate immune response                                           | [11]   |
| p65-RelA, transcription initiation factor TFIID subunit 4B                     | Nuclear factor of kappa light polypeptide gene enhancer in B-cells NF-kB complex                          | Suppression of NF-kB response                                                                       | [112]  |
| TATA-binding protein (TBP) TRIF, toll-like receptor adaptor molecule 1        | Nucleus/Transcription factor                                                                              | May inhibit RNA polymerase II                                                                        | [9,113] |
|                                                                                | Cytoplasm: signalosome                                                                                    | May suppress the types I and III IFN signalling and apoptosis                                        | [13,108] |

---

Copyright © 2016 John Wiley & Sons, Ltd. Rev. Med. Virol. 2016; 26: 251–267. DOI: 10.1002/rmv
METHODS TO IDENTIFY NEW CELLULAR SUBSTRATES FOR ENTEROVIRAL PROTEASES

Given that it has been noted that enteroviral proteases can contribute to disease pathology (e.g. cleavage of dystrophin in the heart muscle), it is possible that other enteroviral diseases are also associated with proteolytic activities of 2A\textsuperscript{pro} and/or 3C\textsuperscript{pro}. The identification of additional host-cell proteins that are targeted by the proteases may thus lead to the identification of novel disease mechanisms. Because enteroviruses are able to cause diverse diseases affecting different tissues and organs, it may also be of relevance to understand how these proteases act in specific tissues and cells.

There are numerous approaches that have been used to study the cellular targets of 2A\textsuperscript{pro} and 3C\textsuperscript{pro}. These include infection of cells or tissues, for example, [108,118,123–125], selective overexpression of viral proteases by transfection, for example, [7,108], transgenic techniques [60], a variety of \textit{in vitro} assays, in which the proteases have been incubated with cell lysates, for example, [108,118,126], and \textit{in silico} prediction of the cleavage sites based on amino acid sequences and composition of potential target proteins [76]. To analyse whether the experimental approaches result in cleavage by enteroviral enzymes, Western blotting is a frequently used method. With Western blotting, it is possible to observe the appearance of cleavage products and/or a decrease in the concentration of the potential target proteins (e.g. Figure 5). However, because antibodies may not recognize the produced fragments, this analysis can be cumbersome. Transfection studies have been used to reveal the protein responsible for the effects observed in the infected cells. Nevertheless, when conducting transfection studies to overexpress a selected viral protein, a caveat may be that the function of the viral protein might be dependent on other viral proteins, for example, [100]. Also, it must be taken into account that the protease precursors could have different protein targets compared to mature proteases.

The technical limitations mentioned in the preceding text may provide an explanation for the contradictory reports in the literature. One study indicated that the 3C\textsuperscript{pro} of coxsackievirus B3 can cleave MAVS [13], while other studies suggest that this cleavage is mediated by 2A\textsuperscript{pro} [4,108]. Enterovirus infections can also activate endogenous proteases including caspases, which also cleave cellular proteins. Barral \textit{et al.} [10] showed that poliovirus-induced apoptosis during the course of infection correlates with the cleavage of MDA5. This cleavage also appeared after the cells were treated with puromycin, an inducer of apoptosis. Thus, activation of endogenous proteases may result in the erroneous identification of protein targets for 2A\textsuperscript{pro} and 3C\textsuperscript{pro}.

Another drawback in the analyses described in the preceding text is that they are all hypothesis-driven. Researchers identify a protein of interest and address whether it is affected by an enterovirus-encoded protease. The outcome is that only one or a few cellular proteins are studied at the time. In order to overcome this shortage, a proteome-wide approach was presented by Weng \textit{et al.} [106], who identified new 3C\textsuperscript{pro} substrates using nuclear extracts that were treated with the 3C\textsuperscript{pro} \textit{in vitro}. The treated lysates were analysed with the combination of 2D electrophoresis and mass spectrometry. They identified eight novel substrates for 3C\textsuperscript{pro}, out of which they analysed the cleavage of stimulation factor 64 in more detail.

Newer methodologies in quantitative proteomics have recently been used to study how enteroviruses affect the host-cell proteome [124,125], and such methods may also be applied to identify new
protease targets [127]. A potential disadvantage with these type of analyses is that they are restricted to the proteins expressed by the infected cell, and will not provide a simultaneous analysis of the whole human proteome.

CLEAVAGE PREDICTIONS USING IN SILICO ANALYSIS TECHNIQUES, BIOINFORMATICS

The sequences and structures of the viral polyproteins, as well as their identified cellular targets, may form the basis for the prediction of novel cellular protein substrates. The most comprehensive work completed to predict new cleavage sites of the entero-viral proteases $2\text{A}^{\text{pro}}$ and $3\text{C}^{\text{pro}}$ has been performed by Blom et al. [76] through the use of a neural network algorithm for prediction. In their study, they used a collection of known cleavage sites to teach the algorithm how to predict the potential cleavage sites. The algorithm scores amino acid sequences for potential cleavage sites based on two calculated parameters, the first being sequence specificity, and the second being surface accessibility. The algorithm is published and available as a free tool on the Internet: NetPicoRNA Server (http://www.cbs.dtu.dk/services/NetPicoRNA/).

NetPicoRNA server seems, however, to underestimate the number of $2\text{A}^{\text{pro}}$ cleavage targets, as for example, Nup98 is not recognized as a potential candidate, while the number of $3\text{C}^{\text{pro}}$ cleavage targets may be overestimated. At the time of the publication of the server (1996), only a limited number of cellular substrates were known, which may have caused a bias to certain kind of cleavage sites. In addition, the surface accessibility prediction was not based on resolved 3D structures, but on ab initio primary sequence analysis and the amino acid compositions of the proteins. Many new substrates have been identified since 1996 (Tables 1 and 2, Tables S1 and S3, and references therein), and a large amount of the human proteome 3D structural data is now available [128]. Indeed, by the end of year 1996, the number of the structures reported in the PDB [129] was 5915, while from 1997 to 2014, this number increased to above 93 000. Therefore, it may be worth revising both the sequence specificity, as well as the surface accessibility predictions, with an aim to develop an improved algorithm that can be useful in identifying novel substrates. Such work is ongoing in our laboratories.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Enteroviruses are important human pathogens whose manifestations range from subclinical infections to severe life-threatening diseases. Hospital visits and symptomatic treatments for the severe infections are costly, and it is clear that a better understanding for the complex disease mechanisms underlying entero-virus-mediated diseases would lead to more efficacious treatments. A few disease mechanisms have been identified, and in the near future, additional ones are likely to be discovered by research teams that integrate many scientific disciplines such as bioinformatics, molecular biology, proteomics and the use of patient materials. Several pathological mechanisms may be explained by the activity of the viral proteases $2\text{A}^{\text{pro}}$ and $3\text{C}^{\text{pro}}$. Some effects, such as the shutting down of host-cell protein synthesis, immune evasion, as well as the hijacking of the cellular machinery to favour virus propagation, may be common to most entero-viruses. However, especially in the persistent types of infections when the production of viral proteins, including proteases $2\text{A}^{\text{pro}}$ and $3\text{C}^{\text{pro}}$, continues for months or years, the degradation of host proteins may be virus- and tissue-specific and may lead to more selective pathological processes (e.g. myocarditis and the development of dilated cardiomyopathy). Currently, there are only a limited number of studies that have addressed the role of the proteases in a tissue-specific manner. Better tools to globally identify and verify protease targets should assist in the identification of novel cellular protein substrates without limitations to particular cell types. Overall, this should provide a better understanding of how the proteases, in concert with other viral proteins, contribute to the induction of different diseases. Such information will also be of immediate importance for the development of novel drugs, including protease inhibitors, to prevent and treat diseases caused by enteroviruses. New prediction methods and proteome-wide approaches are critical for the successful completion of this goal.

CONFLICT OF INTEREST

The authors have no competing interests.

ACKNOWLEDGEMENTS

The authors are very grateful to Ms Sabina Kapell for the artwork (Figure 1) and Dr Virginia Stone.
REFERENCES

1. Jacobs SE, Lamson DM, St George K, et al. Human rhinoviruses. Clinical Microbiology Reviews 2013; 26: 135–162.
2. Tapparel C, Siegrist F, Petty TJ, et al. Picornavirus and enterovirus diversity with associated human diseases. Infection, Genetics and Evolution 2013; 14: 282–293.
3. Hofer D, Alidjnouni EK. Enteroviral pathogenesis of type 1 diabetes: queries and answers. Current Opinion in Infectious Diseases 2013; 26: 263–269.
4. Feng Q, Langereis MA, Lork M, et al. Enterovirus 2Apro targets MDA5 and MAVS in infected cells. Journal of Virology 2014; 88: 3369–3378.
5. Lei X, Xiao X, Xue Q, et al. Cleavage of interferon regulatory factor 7 by enterovirus 71 3C suppresses cellular responses. Journal of Virology 2013; 87: 1690–1698.
6. Wang B, Xi X, Lei X, et al. Enterovirus 71 protease 2Apro targets MAVS to inhibit anti-viral type I interferon responses. PLoS Pathogens 2013; 9: e1002321.
7. Lu J, Yi L, Zhao J, et al. Enterovirus 71 disrupts interferon signaling by reducing the level of interferon receptor 1. Journal of Virology 2012; 86: 3767–3776.
8. Lamphhear BJ, Yan R, Yang F, et al. Mapping the cleavage site in protein synthesis initiation factor elF-4 gamma of the 2A proteases from human coxsackievirus and rhinovirus. Journal of Biological Chemistry 1993; 268: 19200–19203.
9. Clark ME, Lieberman PM, Berk AJ, et al. Direct cleavage of human TATA-binding protein by poliovirus protease 3C in vivo and in vitro. Molecular and Cellular Biology 1993; 13: 1232–1237.
10. Barral PM, Morrison JM, Drahos J, et al. MDA-5 is cleaved in poliovirus-infected cells. Journal of Virology 2007; 81: 3677–3684.
11. Barral PM, Sarkar D, Fisher PB, et al. RIG-I is cleaved during picornavirus infection. Virology 2009; 391: 171–176.
12. Lei X, Liu X, Ma Y, et al. The 3C protein of enterovirus 71 inhibits retinoid acid-inducible gene I-mediated interferon regulatory factor 3 activation and type 1 interferon responses. Journal of Virology 2010; 84: 8051–8061.
13. Mukherjee A, Morosky SA, Delorme-Axford E, et al. The coxsackievirus B 3C protease cleaves MAVS and TRIF to attenuate host type I interferon and apoptotic signaling. PLoS Pathogens 2011; 7: e1001311.
14. Badorff C, Berkely N, Mehrotra S, et al. Enteroviral protease 2A directly cleaves dystrophin and is inhibited by a dystrophin-based substrate analogue. Journal of Biological Chemistry 2000; 275: 11191–11197.
15. Luo H, Wong J, Wong B. Protein degradation systems in viral myocarditis leading to dilated cardiomyopathy. Cardiovascular Research 2010; 85: 347–356.
16. Barnabei MS, Sjaastad FV, Townsend D, et al. Severe dystrophic cardiomyopathy caused by the enteroviral protease 2A-mediated C-terminal dystrophin cleavage fragment. Science Translational Medicine 2015; 7: 294 ra106.
17. Adams MJ, King AM, Carstens EB. Ratio of coxsackievirus A9 into host cells: implications for the initiation of viral infection. Virology 2002; 298: 171–184.
18. Hofer F, Gruenberger M, Kowalski H, et al. Severe dystrophic cardiomyopathy of coxsackievirus A9 is mediated by a dystrophin-based substrate analogue. Journal of Biological Chemistry 2000; 275: 11191–11197.
19. Hafenstein S, Bowman LD, Chipman PR, et al. Interaction of decay-accelerating factor with coxsackievirus B3. Journal of Virology 2007; 81: 12927–12935.
20. Yamashita Y, Iwashita T, Piraino L, Kalkkinen N, Stanway G, Hovi T. RGD-dependent entry of coxsackievirus A9 into host cells and its bypass after cleavage of VP1 protein by intestinal proteases. Journal of Virology 1991; 65: 4735–4740.
21. Ikeda Y, Tamiya K, Taniyama K, et al. Enterovirus A9 infects host cells: immune responses with alpha v beta 3 integrin, the vitronectin receptor. Virology 1994; 203: 357–365.
22. Ikeda S, Nomoto A. Recent insights into poliovirus pathogenesis. Trends in Microbiology 2001; 9: 501–506.
23. Iwasaki A, Weller R, Mueller S, et al. Immunofluorescence analysis of poliovirus receptor expression in Peyer’s patches of humans, primates, and CD155 transgenic mice: implications for poliovirus infection. Journal of Infectious Diseases 2002; 186: 585–592.
24. Marjomaki V, Pietiainen V, Matilainen H, et al. Internalization of coxsackievirus A9 and CD155 transgenic mouse: implications for poliovirus infection. Journal of Infectious Diseases 2002; 186: 585–592.
25. Guest S, Pilipenko E, Sharma K, et al. Molecular mechanisms of attenuation of the enterovirus 71. Journal of Virology 2010; 84: 3666–3681.
26. Ren J, Wang X, Hu Z, et al. Picornavirus uncoating intermediate captured in atomic detail. Nature Communications 2013; 4: 1292.
27. Guest S, Pilipenko E, Sharma K, et al. Molecular mechanisms of attenuation of the Sabin strain of poliovirus type 3. Journal of Virology 2004; 78: 11097–11107.
28. Lind K, Huhn MH, Flodstrom-Tullberg M. Immunology in the clinic review series; focus on type 1 diabetes and viruses: the innate immune response to enteroviruses and its possible role in regulating type 1 diabetes. Clinical and Experimental Immunology 2012; 168: 30–38.
Pathogenicity of enteroviral proteases

34. Lee YF, Nomoto A, Detjen BM, et al. A protein covalently linked to poliovirus genome RNA. *Proceedings of the National Academy of Sciences of the United States of America* 1977; 74: 59–63.

35. Yogo Y, Wimmer E. Polyanhydrid acid at the 3′-terminus of poliovirus RNA. *Proceedings of the National Academy of Sciences of the United States of America* 1972; 69: 1877–1882.

36. Krausslich HG, Nicklin MJ, Lee CK, et al. Polyprotein processing in picornavirus replication. *Biochimie* 1988; 70: 119–130.

37. Nicklin MJ, Krausslich HG, Toyoda H, et al. Poliovirus polypeptide precursors: expression in vitro and processing by exogenous 3C and 2A proteinases. *Proceedings of the National Academy of Sciences of the United States of America* 1987; 84: 4002–4006.

38. Toyoda H, Nicklin MJ, Murray MG, et al. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. *Cell* 1986; 45: 761–770.

39. Hober D, Sane F, Riedweg K, et al. Viruses and type 1 diabetes: focus on the enteroviruses. Available online: http://www.intechopen.com/books/type-1-diabetes/viruses-and-type-1-diabetes-focus-on-the-enteroviruses (accessed on February 2013).

40. Basavappa R, Syed R, Flore O, et al. Role and mechanism of the maturation cleavage of VP0 in poliovirus assembly: structure of the empty capsid assembly intermediate at 2.9 Å resolution. *Protein Science* 1994; 3: 1651–1669.

41. Suhy DA, Giddings TH Jr, Kirkegaard K. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. *Journal of Virology* 2000; 74: 8953–8965.

42. Pathak HB, Arnold JJ, Wiegand PN, et al. Picornavirus genome replication: assembly and organization of the VPg uridylation ribonucleoprotein (initiation) complex. *Journal of Biological Chemistry* 2007; 282: 16202–16213.

43. Novak JE, Kirkegaard K. Improved method for detecting poliovirus negative strands used to demonstrate specificity of positive-strand encapsidation and the ratio of positive to negative strands in infected cells. *Journal of Virology* 1991; 65: 3384–3387.

44. Plevka P, Perera R, Cardosa J, et al. Crystal structure of human enterovirus 71. *Science* 2012; 336: 1274.

45. Nugent CI, Johnson KL, Sarnow P, et al. Functional coupling between replication and packaging of poliovirus replicon RNA. *Journal of Virology* 1999; 73: 427–435.

46. Pieler T, Pasamontes L, Trolle M, et al. Immunocytochemical localization of capsid-related particles in subcellular fractions of poliovirus-infected cells. *Virology* 1992; 188: 676–684.

47. Inal JM, Jorfi S. Coxsackievirus B transmission and possible new roles for extracellular vesicles. *Biochemical Society Transactions* 2013; 41: 299–302.

48. Robinson SM, Tsueng G, Sin J, et al. Coxsackievirus B exits the host cell in shed microvesicles displaying autophagosomal markers. *PLoS Pathogens* 2014; 10: e1004045.

49. Chehadeh W, Kerr-Conje J, Pattou F, et al. Persistent infection of human pancreatic islets by coxsackievirus B is associated with alpha interferon synthesis in beta cells. *Journal of Virology* 2000; 74: 10153–10164.

50. Jartti T, Lehtinen P, Vuorinen T, et al. Persistent infection of human pancreatic islets by coxsackievirus B is associated with alpha interferon synthesis in beta cells. *Journal of Virology* 2000; 74: 10153–10164.

51. Klingel K, Hohenadl C, Caru A, et al. Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection: quantitative analysis of virus replication, tissue damage, and inflammation. *Proceedings of the National Academy of Sciences of the United States of America* 1992; 89: 314–318.

52. Chia MY, Chiang PS, Chung WY, et al. Epidemiology of enterovirus 71 infections in Taiwan. *Pediatrics and Neonatology* 2014; 282: 255–249.

53. Yip CC, Lau SK, Woo PC, et al. Human enterovirus 71 epidemics: what’s next? *Emerging Health Threats Journal* 2013; 6: 19780.

54. Lang M, Mirand A, Savy N, et al. Acute flaccid paralysis following enterovirus D68 associated pneumonia, France, 2014. *Euro Surveillance* 2014; 19: 20952.

55. Khan F. Enterovirus D68: acute respiratory illness and the 2014 outbreak. *Emergency Medicine Clinics of North America* 2015; 33:e19–e32.

56. Stock I. Hand, foot and mouth disease—more than a harmless “childhood disease”. *Medizinische Monatsschrift für Pharmazeuten* 2014; 37: 4–10; quiz 11-2.

57. Lim BK, Peter AK, Xiong D, et al. Inhibition of coxsackievirus-associated dystrophin cleavage prevents cardiomyopathy. *Journal of Clinical Investigation* 2013; 123: 5146–5151.

58. Massilamany C, Gangapala A, Reddy J. Intricacies of cardiac damage in coxsackievirus B3 infection: implications for therapy. *International Journal of Cardiology* 2014; 177: 330–339.

59. Nomoto A. Molecular aspects of poliovirus pathogenesis. *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences* 2007; 83: 266–275.

60. Wong J, Zhang J, Yanagawa B, et al. Cleavage of serum response factor mediated by enteroviral protease 2A contributes to impaired cardiac function. *Cell Research* 2012; 22: 360–371.

61. Estrella NL, Naya FJ. Transcriptional networks regulating the costamere, sarcocere, and other cytoskeletal structures in striated muscle. *Cellular and Molecular Life Sciences* 2014; 71: 1641–1656.

62. Kim KS, Tracy S, Tapprich W, et al. 5′-terminal deletions occur in coxsackievirus B3 during replication in murine hearts and cardiac myocyte cultures and correlate with encapsidation of negative-strand viral RNA. *Journal of Virology* 2005; 79: 7024–7041.

63. Xiong D, Yajima T, Lim BK, et al. Inducible cardiac-restricted expression of enteroviral protease 2A is sufficient to induce dilated cardiomyopathy. *Circulation* 2007; 115: 94–102.

64. Chapman NM, Kim KS, Drescher KM, et al. 5′ terminal deletions in the genome of a coxsackievirus B2 strain occurred naturally in human heart. *Virology* 2008; 375: 480–491.

65. Seippel J, Guarme A, Bergmann E, et al. The structures of picornaviral proteinases. *Virus Research* 1999; 62: 159–168.

66. Kabsch W, Sander C. Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 1983; 22: 5277–5277.

67. Liebig HD, Ziegler E, Yan R, et al. Purification of two picornaviral 2A proteinases: interaction with eIF-4 gamma and influence on in vitro translation. *Biochemistry* 1993; 32: 7581–7588.

68. Wang QM, Johnson RB, Cox GA, et al. Enzymatic characterization of refolded human rhinovirus type 14 2A protease expressed in Escherichia coli. *Journal of Virology* 1998; 72: 1683–1687.
69. Cai Q, Yameen M, Liu W, et al. Conformational plasticity of the 2A protease from enterovirus 71. Journal of Virology 2013; 87: 7348–7356.
70. Mu Z, Wang B, Zhang X, et al. Crystal structure of 2A protease from hand, foot and mouth disease virus. Journal of Molecular Biology 2013; 425: 4530–4543.
71. Sun Y, Wang X, Yuan S, et al. An open conformation determined by a structural switch for 2A protease from coxsackievirus A16. Protein & Cell 2013; 4: 782–792.
72. Shi J, Wei Z, Song J. Dissection study on the severe acute respiratory syndrome 3C-like protease reveals the critical role of the extra domain in dimerization of the enzyme: defining the extra domain as a new target for design of highly specific protease inhibitors. Journal of Biological Chemistry 2004; 279: 24765–24773.
73. Chen S, Zhang J, Hu T, et al. Residues on the dimer interface of SARS coronavirus 3C-like protease: dimer stability characterization and enzyme catalytic activity analysis. Journal of Biochemistry 2008; 143: 525–536.
74. Costenaro L, Kaczmarska Z, Aran C, et al. Structural basis for antiviral inhibition of the main protease, 3C, from human enterovirus 93. Journal of Virology 2011; 85: 10764–10773.
75. Tan J, George S, Kusov Y, et al. 3C protease of enterovirus 68: structure-based design of Michael acceptor inhibitors and their broad-spectrum antiviral effects against picornaviruses. Journal of Virology 2013; 87: 4339–4351.
76. Blom N, Hansen J, Blaa D, et al. Cleavage site analysis in picornaval polypeptides: discovering cellular targets by neural network. Protein Science 1996; 5: 2203–2216.
77. UniProt Consortium. The Universal Protein Resource (UniProt). Nucleic Acids Research 2008; 36: D190–D195.
78. Crooks GE, Hon G, Chandion JM, et al. WebLogo: a sequence logo generator. Genome Research 2004; 14: 1188–1190.
79. Ramajayam R, Tan KP, Liang PH. Recent development of 3C and 3CL protease inhibitors for anti-coronavirus and anti-picornavirus drug discovery. Biochemical Society Transactions 2011; 5: 1371–1375.
80. Thibault HJ, De Palma AM, Neys J. Combating enterovirus replication: state-of-the-art on antiviral research. Biochemical Pharmacology 2012; 2: 185–192.
81. Guo Y, Wang Y, Cao L, et al. A conserved inhibitory mechanism of a lycorine derivative against enterovirus and hepatitis C virus. Antimicrobial Agents and Chemotherapy 2015; 2: 913–924.
82. Mandadapu SR, Weerasekara PM, Prior AM, et al. Macroyclic inhibitors of 3C and 3C-like proteases of picomavirus, norovirus, and coronaviruses. Bioorganic and Medicinal Chemistry Letters 2013; 13: 3709–3712.
83. Dragovich PS, Prins TJ, Zhou R, et al. Structure-based design, synthesis, and biological evaluation of irreversive human rhinovirus 3C protease inhibitors. Incorporation of P1 lactam moieties as L-glutamate replacements. Journal of Medicinal Chemistry 1999; 7: 1213–1224.
84. Rocha-Pereira J, Nascimento MS, Ma Q, Hilgenfeld R, Neys J, Joehmans D. The enterovirus protease inhibitor rupintrivir exerts cross-genotypic anti-norovirus activity and clears cells from the norovirus replicon. Antimicrobial Agents and Chemotherapy 2014; 8: 4675–4681.
85. Patrick AK, Brothers MA, Maldonado F, et al. In vitro antiviral activity and single-dose pharmacokinetics in humans of a novel, orally bioavailable inhibitor of human rhinovirus 3C protease. Antimicrobial Agents and Chemotherapy 2005; 6: 2267–2275.
86. Hayden FG, Turner RB, Gualtney JM, et al. Phase II randomized, double-blind, placebo-controlled studies of rupintrivir nasal spray 2-percent suspension for prevention and treatment of experimentally induced rhinovirus colds in healthy volunteers. Antimicrobial Agents and Chemotherapy 2003; 12: 9907–9916.
87. Huyu PH, Pithavala YK, Gersten M, Penning CA, Kerr BM. Pharmacokinetics and safety of an antihiviral agent, rupintrivir, in healthy volunteers. Antimicrobial Agents and Chemotherapy 2002; 2: 392–397.
88. van der Linden L, Wolthers KC, van Kuppeveld FJ. Replication and inhibitors of enteroviruses and parechoviruses. Viruses 2015; 8: 4529–4562.
89. De Palma AM, Vliegen I, De Clercq E, Neys J. Selective inhibitors of picomavirus replication. Medicinal Research Reviews 2008; 6: 823–884.
90. Wang J, Fan T, Yao X, et al. Crystal structures of enterovirus 71 3C protease complexed with rupintrivir reveal the roles of catalytically important residues. Journal of Virology 2011; 19: 10021–10030.
91. Zhang X1, Song Z, Qin B, et al. Rupintrivir is a promising candidate for treating severe cases of enterovirus-71 infection: evaluation of antiviral efficacy in a murine infection model. Antiviral Research 2013; 3: 264–269.
92. Lu G, QJ, Chen Z, et al. Enterovirus 71 and coxsackievirus A16 3C proteases binding to rupintrivir and their substrates and antiflu, foot, and mouth disease virus drug design. Journal of Virology 2011; 19: 1310–1311.
93. Sun L, Meijer A, Froeyen M, et al. Antiviral activity of broad-spectrum and enterovirus-specific inhibitors against clinical isolates of enterovirus D68. Antimicrobial Agents and Chemotherapy 2015; 2: 7782–7785.
94. Jang SK, Krasslick HC, Nicklin MJ, et al. A segment of the 5' nontranslated region of onchopomycoriditis virus RNA directs internal entry of ribosomes during in vitro translation. Journal of Virology 1988; 62: 2606–2643.
95. Pelletier J, Sonenberg N. Internal binding of eucaryotic ribosomes on poliovirus RNA: translation in HeLa cell extracts. Journal of Virology 1989; 63: 441–444.
96. Weidman MK, Yalamanchili P, Ng B, et al. Poliovirus 3C protease-mediated degradation of transcriptional activator p53 requires a cellular activity. Virology 2001; 291: 260–271.
97. Yalamanchili P, Weidman K, Dasgupta A. Cleavage of transcriptional activator Oct-1 by poliovirus encoded protease 3Cpro. Virology 1997; 239: 176–185.
98. Yalamanchili P, Harris K, Wimmer E, et al. Inhibition of basal transcription by poliovirus: a virus-encoded protease (3Cpro) inhibits formation of TBP-TATA box complex in vitro. Journal of Virology 1996; 70: 2922–2929.
99. Dingwall C, Laskey RA. Nuclear targeting sequences—a consensus? Trends in Biochemical Sciences 1991; 16: 478–481.
100. Tian W, Cui Z, Zhang Z, et al. Poliovirus 2A (Pro) induces the nuclear translocation of poliovirus 3CD and 3C' proteins. Acta Biochimica et Biophysica Sinica Shangh 2011; 43: 38–44.
101. Lamphear BJ, Rhoads RE. A single amino acid change in protein synthesis initiation factor 4G renders cap-dependent translation resistant to picornavirus 2A proteases. Biochemistry 1996; 35: 15726–15733.
102. Goldstaub D, Gradi A, Bercovitch Z, et al. Poliovirus 2A protease induces apoptotic cell death. Molecular and Cellular Biology 2000; 20: 1271–1277.
SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Table S1. Published enteroviral 2A substrates.
Table S2. Published enteroviral 3C substrates.