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REVIEW

Enteroviral proteases: structure, host interactions and pathogenicity

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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, $2A^{pro}$ and $3C^{pro}$, 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 $2A^{pro}$-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.

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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

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Abbreviations used
$2A^{pro}$, enteroviral protease 2A, $3C^{pro}$, enteroviral protease 3C; HFMD, hand-foot-and-mouth disease; PRR, pattern recognition receptor; VP, viral capsid protein; IgSF, immonoglobulin superfamily; ICAM-1, intracellular adhesion molecule; CAR, coxsackievirus–adenovirus receptor; PVR, poliovirus receptor; DAF, decay accelerating factor; LDL-R, low-density lipoprotein receptor; PTB, polypyrimidine tract-binding protein; $3D^{pol}$, 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.

disease-causing mechanisms and the identification of targets for the development of therapeutic measures. All enteroviruses encode two proteases, 2A ($2A^{pro}$) and 3C ($3C^{pro}$), 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 *Enterovirus*. The genus belongs to the family of Picornaviridae, under the order of Picornavirales, and the genus is divided into twelve species: *Enterovirus* A–H, J and *Rhinovirus* 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 four separate viral capsid proteins (VP1–VP4). Upon infection, the capsid undergoes structural changes, causing the release of the viral genome into the cytoplasm where it undergoes translation by the translation machinery of the host. The enterovirus genome encodes a single open reading frame, resulting in translation of all viral proteins as a single polyprotein.

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 superfamily (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

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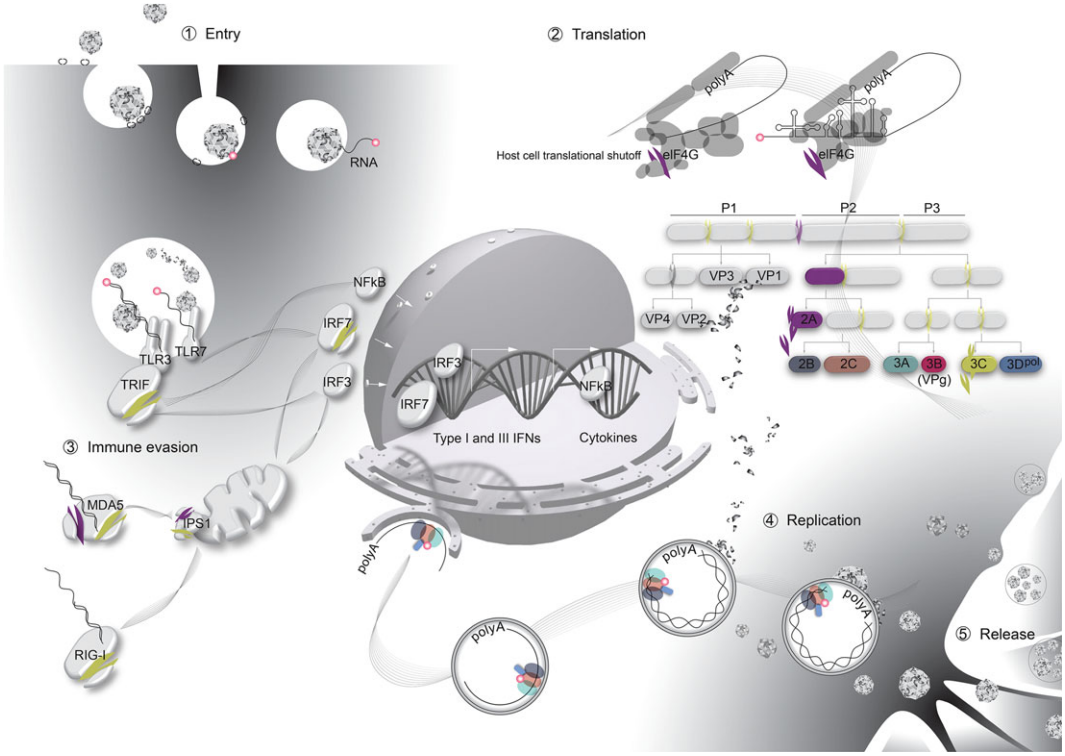

Figure 1. Proposed model of the enterovirus replication cycle. (1) Entry. After attachment to host-cell surface receptors virus is internalized and uncoated, leading to the release of viral RNA into the cytoplasm. (2) Translation. Viral polyprotein is translated and then processed by the $2A^{pro}$ and $3C^{pro}$ proteases. Host-cell translation is also perturbed as a component of the translation machinery (eIF4G) cleaved by $2A^{pro}$. (3) Immune evasion. Host-cell immune response is blunted by proteolysis mediated by viral proteases $2A^{pro}$ and $3C^{pro}$ 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.

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 $2A^{pro}$ and $3C^{pro}$ [36–38].

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 $2A^{pro}$ is translated first, $2A^{pro}$ 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, $3C^{pro}$, 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 $3D^{pol}$ 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

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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 $2A^{pro}$ 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 $2A^{pro}$ 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 $2A^{pro}$-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 $2A^{pro}$ 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 $2A^{PRO}$ AND $3C^{PRO}$

Structural features of enterovirus proteases

The enterovirus proteases $2A^{pro}$ and $3C^{pro}$ are multifunctional cysteine proteases, belonging to the chymotrypsin-related endopeptidase protease family [65] (MEROPS $2A^{pro}$: C03.020 and $3C^{pro}$:

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C03.011). When comparing 2A^{pro} to 3C^{pro}, 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 *Enterovirus* 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^{pro}, 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^{pro}

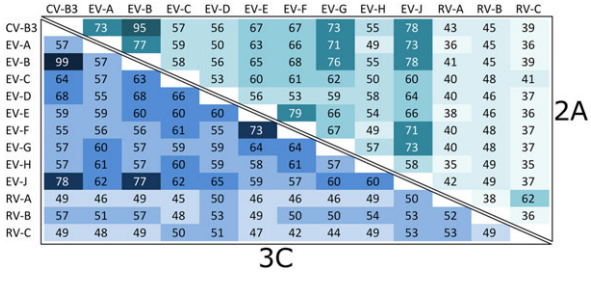

Figure 3. Primary amino acid sequence percentage identity matrix of the enteroviral proteases. The top-right half of the matrix shows sequence identities between the different enteroviral species and coxsackievirus B3 for 2A^{pro}, and the lower-left half for 3C^{pro}. The average sequence conservation between the different species is 53% for 2A^{pro} and 56% for 3C^{pro}. The rhinoviruses show the most sequence divergence with around 35–50% percentage identities for 2A^{pro} and 45–55% for 3C^{pro}.

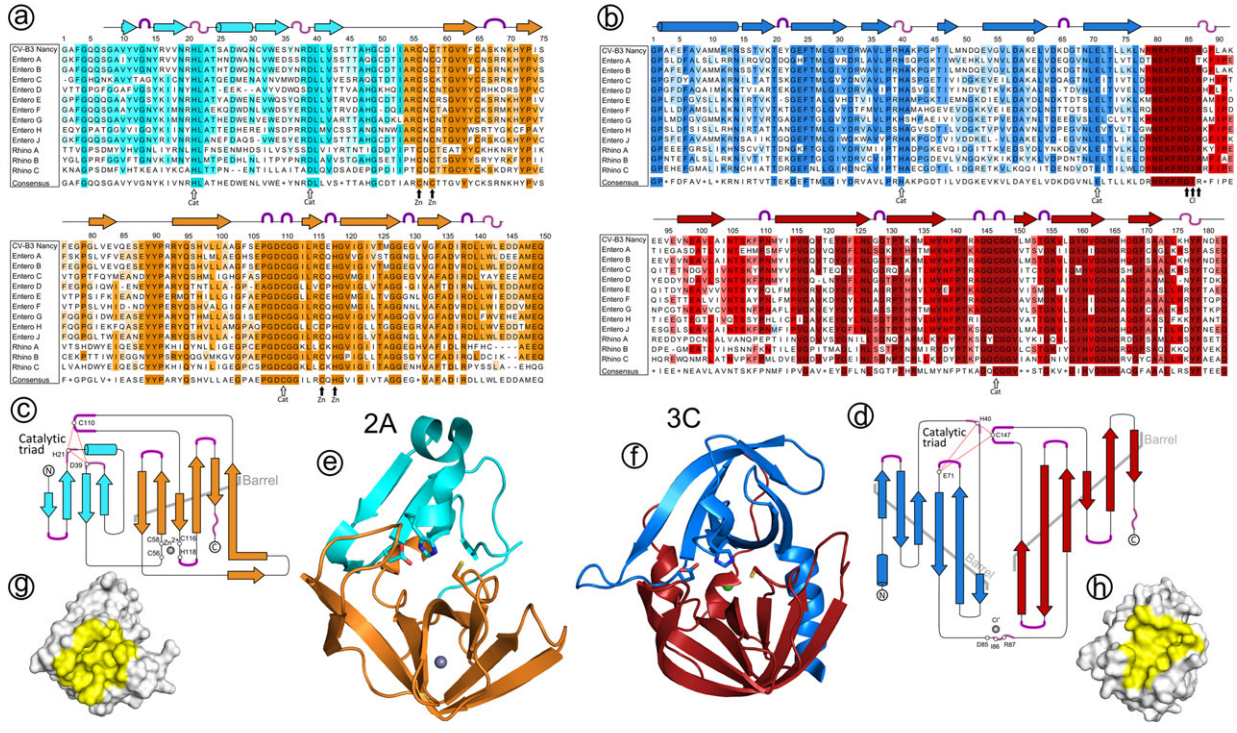

Figure 2. Sequence alignments of 2A^{pro} and 3C^{pro}, their topological structure presentations and 3-dimensional tertiary structures. Orange and cyan colourings are used for 2A^{pro}, and red and blue colourings are used for 3C^{pro}. Panels (a) and (b) show the primary amino acid sequence alignment of 2A^{pro} and 3C^{pro} within the Enterovirus family. The residues of the catalytic triad, as well as the ion-binding residues, are highlighted with arrows underneath the sequences. The secondary structure elements are shown above the alignments (cylinder = alpha-helical structure; arrow = beta-sheet structure; turns in purple; 3/10 helices in pink). Secondary structure assignment was made using DSSP [66]. Panels (c) and (d) show a topological schematic of the proteases. The same visual secondary structure representations are used as in panels (a) and (b). Panel (e) shows a cartoon representation of EV71 2A^{pro} (PDBID: 4FVB). The side chains of the amino acids of the catalytic triad are shown as sticks. Similarly to panel (e), panel (f) shows a cartoon representation of CVB3 3C^{pro} (PDBID: 2VB0). Panels (g) and (h) show surface representations of the proteases, with their active sites highlighted with yellow. In comparison, the active site of 2A^{pro} is more confined and restricted by surrounding structures than the active site of 3C^{pro}.

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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 $2A^{pro}$, and histidine, glutamic acid and cysteine in the case of $3C^{pro}$. 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 $2A^{pro}$, a zinc ion is located in one end of the barrel, bound by three cysteines and one histidine residue. For $3C^{pro}$, 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 $2A^{pro}$. Liebig *et al.* found that HRV2 $2A^{pro}$ showed a dimeric state in gel filtration analysis, while CVB4 $2A^{pro}$ was found to be monomeric [67]. In another study, $2A^{pro}$ from HRV14 was found to be monomeric by gel filtration analysis [68]. In a study by Cai *et al.*, EV71 $2A^{pro}$ 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 $2A^{pro}$ and found a monomer in the asymmetric unit [70]. In another recent study of CVA16 $2A^{pro}$, 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 $3C^{pro}$ 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 $3C^{pro}$ proteases assembled as dimers. For example, $3C^{pro}$ 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 $2A^{pro}$ and $3C^{pro}$ proteases are able to cleave (or not), has not been established or studied comprehensively. To date, the amino acid residues P_4, P_2, P_1, P_1' and P_2' are recognized as being important determinants for the sequence specificity of enteroviral proteases (Figure 4) [65,76]. For the substrate recognition of $2A^{pro}$, the most important residue is P_1', which is exclusively a glycine. Following P_1' in order of importance are P2, occupied mainly by threonine and asparagine; P_2', occupied by proline, alanine and phenylalanine; and P_4, occupied most frequently by leucine or threonine. For $3C^{pro}$, the residues P_1 and P_1' show the least amount of variance in the substrate sequence. The preferred residues for these positions are glutamine or glutamate for P_1, and glycine, asparagine or serine for P_1'. In addition, the most common residue is alanine in position P_4 and proline in position P_2'. The most obvious feature for determining the substrate specificity of both $2A^{pro}$ and $3C^{pro}$ is the strong conservation of the glycine residue in position P_1' [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, $3C^{pro}$ 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

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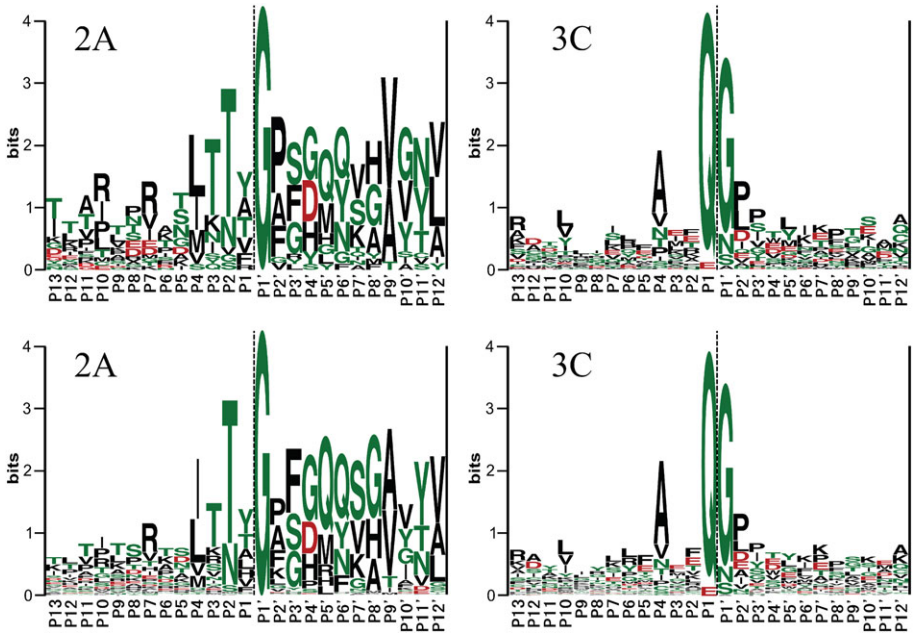

Figure 4. The substrate sequence LOGOs of the enteroviral proteases published by Blom *et al.* 1996 [76] (upper panels) (reprinted with permission from John Wiley and Sons) and new logos based on a larger substrate pool (lower panels; Nurminen *et al.* manuscript) show the most conserved positions of the substrate sequences around the cleavage site. Hydrophilic residues are shown in green colour, and hydrophobic residues are shown in black colour. Negatively charged residues are coloured red. The lower panel logos were created using all currently available enteroviral polyprotein sequences in the Uniprot database [77]. Duplicate sequences were removed to avoid bias towards sequences with multiple entries. The logos were generated using WebLogo [78]. Left panels: The most important recognition sites for $2A^{pro}$ in order of lowest variability are at locations P1′, P2, P2′, P4 and P3. The relatively low variability to the right of P2′ can be a result of the sequence being a functional part of $2A^{pro}$ itself, as the protease cleaves its own N-terminal end free from the polyprotein by in cis cleavage. Right panels: The most important recognition sites for $3C^{pro}$ in order of lowest variability are at locations P1, P1′, P4 and P2′.

distantly related viruses. Such inhibitor candidates include pyrazole compounds that target $3C^{pro}$ from different enteroviruses as well as coronavirus protease homologues of $3C^{pro}$ [79], microcyclic inhibitors against enterovirus $3C^{pro}$ and noro- and SARS-coronavirus $3C^{pro}$ homologues [82]. Additionally, a lycorine derivative, 1-acetyllycorine, has been shown to inhibit EV71 $2A^{pro}$ 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 $3C^{pro}$ 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 $2A^{pro}$ and $3C^{pro}$ 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 $2A^{pro}$ 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

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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 $2A^{pro}$-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, $2A^{pro}$ targets several nuclear pore complex (NPC) proteins like Nup62, -98 and -153 [114,115]. This

Table 1. Examples of published enteroviral 2A substrates

Target protein	Virus	Refseq/ UniProtKB AC	Gene	Cleavage site (sequence)
Dystrophin	CVB3	NP_000100/P11532	*DMD*	$PGLTTI_{2434}$-GASP
eIF4GI	CVB4 Polio	NP_886553/Q04637	*EIF4G1*	$TTLSTR_{681}$-GPPR
Melanoma differentiation-associated protein 5(MDA5)	EV71	NP_071451/Q9BYX4	*IFIH1*	$RTVATS_{53}$-GNMQ[a]
Interferon (α, β and ω) receptor 1	EV71	NP_000620/P17181	*IFNAR1*	$RSDESV_{56}$-GNVT[a] $RVQASD_{311}$-GNNT[a]
Nucleoporin 62	Polio RV16	NP_001180286/P37198	*NUP62*	$PATQTT_{72}$-GFTF[a] $ATITST_{217}$-GPSL[a] $TPVTTA_{246}$-GAPT[a] $EHLNTS_{461}$-GAPA[a]
Nucleoporin 98	Polio HRV2 HRV16	NP_005378/P52948	*NUP98*	$VGSTLF_{374}$-GNNK $KALQTT_{552}$-GTAK[a]
Nucleoporin 153	Polio	NP_001265138/P49790	*NUP153*	$SCTVTT_{781}$-GTLG[a] $QTTSST_{1266}$-GTAV[a] $NNTTTS_{1287}$-GFGF[a]
Serum response factor	CVB3	NP_003122/P11831	*SRF*	$TVLKST_{326}$-GSGP

Equal to UniprotKB sequence P52948 amino acid G569.
[a]Predicted, unconfirmed cleavage site (Nurminen *et al*. Manuscript in preparation).

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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].

The role for $2A^{pro}$ and $3C^{pro}$ 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

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 differentiation-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	[7]
Nucleoporin 62	—	—	[103,104]
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]

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Table 2. Examples of published enteroviral 3C substrates

Target protein	Virus	Refseq/ UniProtKB AC	Gene	Cleavage site (sequence)
Cleavage stimulation factor (Cst-64)	EV71	NP_001293138/P33240	*CSTF2*	$LMQASM_{250}$-QGGV one or more of glycines: 483, 496, 505, 510 and 515
CRE-binding protein/cyclic AMP-responsive element-binding protein 1	Polio	NP_004370/P16220	*CREB1*	$YIAITQ_{187}$-GGAI
Interferon regulatory factor 7, IRF7	EV71	NP_001563/Q92985	*IRF7*	$LLQAVQQ_{189}$-SCLA
Mitochondrial antiviral signalling protein (MAVS)	CVB3	NP_065797/Q7Z434	*MAVS*	$PVQETQ0_{148}$-APES
Nucleoporin 62	RV14	NP_714941/P37198	*NUP62*	Many potential cleavage sites
Nucleoporin 153	RV14 RV16	NP_001265138/P49790	*NUP153*	Many potential cleavage sites
Octamer binding transcription factor	Polio RV16	NP_002688/P14859	*POU2F1* (*OCT1*)	$KLGFTQ_{329}$-GDVG
Probable ATP-dependent RNA helicase, RIG-I	Polio echo1 RV16	NP_055129/O95786	*DDX58*	$KMIQTR_{728}$-GRGR[a]
p65-RelA, transcription initiation factor TFIID subunit 4B	Polio	NP_001230913/Q04206	*RELA*	$QQLLNQ_{480}$-GIPV
TATA-binding protein (TBP)	Polio	NP_003185/P20226	*TBP*	$GLASPQ_{18}$-GAMT
TRIF, toll-like receptor adaptor molecule 1	CVB3	NP_067681/Q86XR7	*TICAM1* (*TRIF*)	$TPFALQ_{190}$-TINA

[a]Predicted, unconfirmed cleavage site (Nurminen *et al*. Manuscript in preparation).

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

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 1	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-κB complex	Suppression of NF-κB response	[112]
TATA-binding protein (TBP)	Nucleus/Transcription factor	May inhibit RNA polymerase II	[9,113]
TRIF, toll-like receptor adaptor molecule 1	Cytoplasm: signalosome	May suppress the types I and III IFN signalling and apoptosis	[13,108]

the proteases to escape recognition by the immune system. It has been shown that both $2A^{pro}$ and $3C^{pro}$ 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 $3C^{pro}$ and/ or $2A^{pro}$ [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 $2A^{pro}$ acts directly on the interferon receptor 1, reducing its expression and thereby impairing the efficacy of IFN as a treatment against infection [7].

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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 $2A^{pro}$ and/or $3C^{pro}$. 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 number of approaches that have been used to study the cellular targets of $2A^{pro}$ and $3C^{pro}$. 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 *in vitro* assays, in which the proteases have been incubated with cell lysates, for example, [108,118,126], and *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 texts may provide an explanation for the contradictory reports in the literature. One study indicated that the $3C^{pro}$ of coxsackievirus B3 can cleave MAVS [13], while other studies suggest that this cleavage is mediated by $2A^{pro}$ [4,108]. Enterovirus infections can also activate endogenous

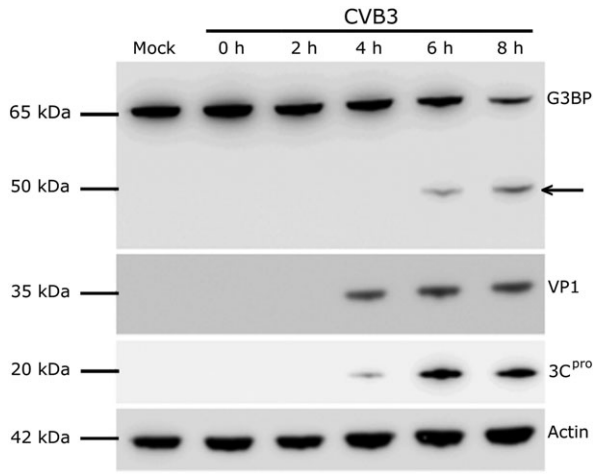

Figure 5. Infection of HeLa cells with coxsackievirus B3 (CVB3) results in the proteolytic cleavage of Ras GTPase-activating protein-binding protein 1 (G3BP1). HeLa cells were infected at a MOI of 20 with CVB3, (mock control sample treated with media alone). At each time-point, the cells were lysed and the expression of G3BP1 and viral proteins VP1 and $3C^{pro}$ were analysed by Western blot. The arrow indicates an accumulation of G3BP1 cleavage product 6 h post infection. Actin was used as a loading control.

proteases including caspases, which also cleave cellular proteins. Barral *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 $2A^{pro}$ and $3C^{pro}$.

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 *et al*. [106], who identified new $3C^{pro}$ substrates using nuclear extracts that were treated with the $3C^{pro}$ *in vitro*. The treated lysates were analysed with the combination of 2D electrophoresis and mass spectrometry. They identified eight novel substrates for $3C^{pro}$, 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

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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 enteroviral proteases $2A^{pro}$ and $3C^{pro}$ 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 $2A^{pro}$ cleavage targets, as for example, Nup98 is not recognized as a potential candidate, while the number of $3C^{pro}$ 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 enterovirus-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 $2A^{pro}$ and $3C^{pro}$. 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 enteroviruses. However, especially in the persistent types of infections when the production of viral proteins, including proteases $2A^{pro}$ and $3C^{pro}$, 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.

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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.