Viral security proteins: counteracting host defences

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Abstract | Interactions with host defences are key aspects of viral infection. Various viral proteins perform counter-defensive functions, but a distinct class, called security proteins, is dedicated specifically to counteracting host defences. Here, the properties of the picornavirus security proteins L and 2A are discussed. These proteins have well-defined positions in the viral polyprotein, flanking the capsid precursor, but they are structurally and biochemically unrelated. Here, we consider the impact of these two proteins, as well as that of a third security protein, L*, on viral reproduction, pathogenicity and evolution. The concept of security proteins could serve as a paradigm for the dedicated counter-defensive proteins of other viruses.

All bona fide RNA viruses encode at least two proteins, a capsid protein and an RNA-dependent RNA polymerase (RdRP). In this article, we do not consider ‘abnormal’ RNA viruses such as hepatitis delta virus, which does not possess its own replication machinery and borrows a capsid from another virus, or the capsid-less narnaviruses, which encode only an RdRP; hepatitis delta virus and narnaviruses are closer to viroids and plasmids, respectively, than to fully fledged RNA viruses. RNA viruses encoding only a capsid and an RdRP are known to infect protists such as Giardia spp.†. Some RNA phages and fungal RNA viruses express only three proteins. However, most RNA viruses do not rely on such a scant protein repertoire. The proteomes of the majority of RNA viruses comprise up to 12 different proteins, with the genomes of some coronaviruses encoding nearly 30 proteins.

To infect, viruses must reach an appropriate intracellular environment and, if necessary, adapt this environment to their own requirements. Thus, viral infection requires not only replication but also interactions with host defences. To carry out these tasks, RNA viruses have only a few proteins at their disposal, with the available protein arsenal being limited by the genome size. As the proteomes of these viruses are strikingly small and specific viral functions generally require more than one protein, most proteins encoded by RNA viruses are multifunctional. At the same time, however, there is a certain division of labour between viral proteins. We consider some aspects of this problem using, as an example, the picornaviruses, a large family of small animal viruses with a medium-sized, single-stranded, positive-sense RNA genome (BOX 1). Although they share a common genome organization, these viruses exhibit sufficient genetic variation to be separated into at least 12 distinct genera (BOX 2). The family includes important human and animal pathogens that cause a range of disorders, including poliomyelitis, foot-and-mouth disease, the common cold, gastroenteritis, hepatitis, meningitis, myocarditis and uveitis. In addition to acute diseases, these viruses can also cause chronic persistent disorders.

Of the 12 ‘mature’ (fully processed) picornaviral proteins (BOX 1), the most ancient group includes the capsid proteins and a set of conserved proteins considered to be picornavirus signature proteins, comprising the RdRP (3D<sup>pol</sup>), the primer for RNA synthesis (VPg), a protease (3C<sup>pro</sup>) and an ATPase (2C<sup>pol</sup>). The capsid and signature proteins are indispensable for viral viability. Two less conserved proteins, 2B and 3A, are also essential for viability but are less directly involved in viral reproduction. The main functions of these two proteins are to target replicative proteins to the correct destinations and aid in the creation of a suitable replicative niche.

Finally, two other non-structural proteins that flank the capsid precursor in the polyprotein molecule, the leader (L) and 2A proteins, constitute a distinct group, although they have no common structural or biochemical features. They are the most variable among the picornavirus proteins, and some viruses even lack L altogether (FIG. 1; TABLES 1, 2). This group also includes the so-called L* protein, which is encoded in a different reading frame of the RNA of certain cardioviruses. Functionally, these proteins (L, L* and 2A) have been characterized in some
Box 1 | Picornavirus genes and proteins

Picornaviruses possess a single-stranded positive-sense RNA genome (see the figure) comprising approximately 7,000–9,000 nucleotides. The RNA is packaged into an icosahedral capsid usually composed of four distinct proteins (VP1–VP4), but in some viruses VP4 and VP2 are fused (forming VP0). After productive contacts with specific cellular receptors, which differ between viruses, the genome is uncoated and enters the cytoplasm, where the main steps of viral reproduction occur. The viral RNA, which contains a single ORF (with one exception described in the main text), is translated in a cap-independent manner into a 2,200–2,500 amino acid polypeptide, which is eventually processed by limited proteolysis into a dozen ‘mature’ proteins. These proteins include: capsid proteins; an RNA-dependent RNA polymerase (3Dpol); a protein (Vpg, or 3B) that serves as a primer for the initiation of RNA synthesis; an ATPase with a conserved superfamily 3 helicase motif (2C(Pro)) and an essential but poorly defined role in viral RNA replication; a chymotrypsin-like protease (3Cpro), which, as the mature protein or as a precursor, is a major factor in polyprotein processing; two hydrophobic membrane-binding proteins (2B and 2A) that participate in the generation of a virus-friendly environment; and, flanking the precursor of the capsid proteins, one or two highly variable proteins (L and 2A), the structure and functions of which are the subject of this Review. The entire coding region of the RNA is surrounded with untranslated regions (UTRs) harbouring regulatory elements that control viral translation (for example, the internal ribosome entry site (IRES) and replication (the origins oriL and oriR), and this entire RNA sequence is flanked by the covalently linked viral protein Vpg and a poly(A) tract at the 5’ and 3’ termini, respectively. There are four structurally and functionally distinct types of IRES and many types of oriL and oriR.

![Diagram of picornavirus genes and proteins](image)

![Diagram of picornavirus genes and proteins](image)

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Picornavirus taxonomy

Members of the Picornaviridae family are small (~30 nm), non-enveloped animal viruses with similar genome organizations and mechanisms of reproduction (Box 1). This family contains important human and animal pathogens, studies of which have made crucial contributions to our understanding of the molecular biology of viruses and the mechanisms of virus–cell interactions. The first animal virus (foot-and-mouth disease virus; FMDV) and the first human virus (poliovirus) to be discovered belong to this family.

The current official classification of picornaviruses (see the International Committee on Taxonomy of Viruses) includes 12 genera: Aphthovirus (for example, FMDV), Avihepadnavirus (for example, duck hepatitis A virus), Cardioivirus (including important model viruses such as encephalomyocarditis virus, mengovirus and Theiler’s murine encephalomyelitis virus, as well as human and animal pathogenic viruses such as coxsackieviruses, echoviruses and other ‘numbered’ enteroviruses, as well as rhinoviruses, the causative agents of the common cold), Erbovirus (equine rhinitis B virus), Hepatovirus (hepatitis A virus), Kobuvirus (for example, Aichi virus, which induces gastroenteritis in humans, as well as some animal pathogens), Parechovirus (the causative agents of various human diseases, particularly in children; also isolated from rodents), Sapovirus (involving an avian, a porcine and a simian pathogen), Senecavirus (Seneca Valley virus, a candidate oncolytic agent), Teschovirus (a porcine pathogen) and Tremovirus (avian encephalomyelitis virus). In addition, there are two candidate genera, not yet officially approved, that are associated with human diarrhoea: Cosavirus and Klassevirus. Finally, seal picornavirus type 1 is a distinct virus not assigned to any of the above genera.

Several new picornavirus genera have been identified recently, and it seems plausible that this number will grow, particularly with the breakthroughs in sequencing techniques and the achievements of metagenomics.

Cap-dependent translation

The mode of translation for which initiation is dependent on the presence of the so-called cap structure (m‘GpppN) at the 5′ end of an mRNA. Specific cap-binding proteins (translation initiation factors) recruit the ribosome to the 5′ end of the mRNA, and the ribosome scans the template until it encounters the initiation codon. This translation mode is exploited by most mRNAs in eukaryotic cells.

Poly(A)-binding protein 1

A protein that binds to the 3′ poly(A) tail of eukaryotic mRNAs and is required to cap-dependent translation initiation factors on the other hand. This dual binding results in a non-covalent circulation of the mRNA template, which is accompanied by a significant increase in translation efficiency.

Apart from the NPG(P)-containing peptides, 2A proteins of cardioviruses, aviparoviruses, Ljungan virus and seal picornavirus type 1 contain distinct but poorly characterized sequences. The cardioivirus encephalomyocarditis virus (EMCV) encodes a 2A protein with RNA-binding affinity. The bipartite 2A of Ljungan virus, the tripartite 2A of aviparoviruses and the NPG(P)-lacking 2A proteins of kobuviruses, tremoviruses and some parechoviruses all have a ~140–150-residue H-NC domain, which contains a histidine with a downstream asparagine-cysteine dipeptide and a putative transmembrane domain. The observation that a similar H-NC domain is present in certain cellular tumour suppressors suggests that these viral proteins are also involved in the control of host activities. Between the NPG(P) and H-NC motifs, 2A of the aviparovirus duck hepatitis A virus possesses an additional moiety that contains the so-called AIG1 domain, which is also found in representatives of the Ras-like GTPase superfamily.

Cardiovirus L proteins, which are devoid of any enzymatic activity and exhibit noticeable intra-genus variability, contain a non-classical but functional zinc finger (Cys-His-Cys) motif and a downstream acidic motif that, in some of these L proteins, contains potential phosphorylation sites. Certain strains of Theiler’s murine encephalomyelitis virus (TMEV) are unique among picornaviruses in expressing a functional protein, L′, that is encoded in an alternative translation frame that starts within the L coding sequence, goes through the VP4 coding sequences and terminates in the VP2 coding sequence of the main reading frame. The 2A and L proteins of other picornaviruses neither contain easily identifiable amino acid motifs nor have known specific biochemical activities.

Effects on general host metabolism

Security proteins contribute substantially to the shut-off of host macromolecular synthesis that occurs in response to infection with many picornaviruses. One could perhaps argue that such effects of security proteins contradict the key proposal of this Review that these proteins are dedicated to counter-defensive functions. However, as already mentioned, inflicting harm on their hosts does not bring viruses any benefits per se. The only reason (or at least, the main reason) why viruses evolved the ability to damage infected cells is their need to incapacitate the cellular defensive machinery. This machinery includes several specific mechanisms (such as innate and adaptive immunity), the implementation of which requires general cellular functions such as translation, transcription and controllable nucleocytoplasmic trafficking. Therefore, virus-induced impairment of these all-purpose metabolic functions can be regarded as a component of the viral counter-defensive strategy.

The effect of security proteins on cap-dependent translation of cellular mRNA is particularly important. 2A′′′ from diverse enteroviruses and L′′′ from alphavirus cleave eukaryotic translation initiation factor elf4F4G. Interestingly, enterovirus L′′′ does not seem to cleave elf4F4G and does not trigger translational shut-off. Poly(A)-binding protein 1 (PABP1; also known as
cytoplasmic PABP), a host protein that is also involved in translational control, is another target of enterovirus 2A [REFS 40, 41].

Security proteins can inhibit host translation by mechanisms other than proteolysis. Mutations of cardiovirus 2A (which is not a protease) alleviate virus-induced translational shut-off [REFS 42, 43]. It is thought that association of cardiovirus 2A with ribosomes [REFS 44, 45], which seems to take place in the nucleolus [REFS 46, 47] and possibly occurs through the RNA-binding activity of 2A [REFS 48, 49], might contribute to preferential use of the internal ribosome entry site (IRES)-dependent viral templates. Cardiovirus 2A was also reported to mediate translational shut-off [REFS 50, 51]. However, this effect could largely be due to L-mediated inhibition of nuclear export of mRNA rather than to inhibition of translation per se [REFS 52, 53]. On the basis of ectopic expression experiments, hepatitis A virus 2A has also been implicated in inhibition of cap-dependent translation [REFS 54, 55], but the relevance of this observation is uncertain, as hepatitis A virus does not exert translational shut-off.

The effects of security proteins on cellular transcription are less well studied, although synthesis of the mRNA for cytokines and chemokines is inhibited in certain cases (see below). Individually expressed poliovirus 2A [REFS 56, 57] was reported to cleave the general transcription factors TATA-box-binding protein [REF 58] and cyclic AMP-responsive element-binding protein 1 [REF 59], but the biological significance of these effects is debatable [REFS 60, 61]. Poliovirus 2A [REFS 62, 63] cleaves GEMIN3 (also known as DDX20), a protein that is involved in the formation of spliceosomes [REFS 64, 65]. Cardiovirus 2A has also been implicated in virus-triggered inhibition of host transcription [REFS 66, 67], but this effect was not investigated further. Foot-and-mouth disease virus (FMDV) L [REFS 55, 56] and human parecho virus 2A [REFS 68, 69] accumulate in the nucleus during the course of infection and as a result of ectopic expression, respectively, but their nuclear effects are unknown. Enterovirus 2A [REFS 70, 71] cleaves some cytoskeletal proteins, such as cytokeratin 8 [REF 58] and dystrophin, a protein that connects the cytoskeleton to the plasma membrane [REFS 72, 73].

The security proteins of several picornaviruses profoundly affect nucleocytoplasmic transport in infected cells. The targets of enterovirus 2A [REFS 74, 75] include nucleoporins, which are nuclear pore components that control nucleocytoplasmic exchange [REFS 76, 77]. As a result, bidirectional

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**Figure 1 | Leader and 2A proteins of picornaviruses.** The organization of an ‘idealized’ picornaviral polyprotein is shown, with specific viral leader (L) and 2A proteins given below; protein sizes are not to scale but give approximate relative lengths. There is great variability in L and 2A proteins. Multiple L and 2A proteins are known for some viral genera. Several picornaviruses do not possess L but have large 2A proteins. Cosaviruses contain no L and only a very short 2A. Other viruses (for example, some sapeloviruses) possess an unusually long L (we propose that in this case it might correspond to at least two separate polypeptides; see Supplementary information S1 (figure)) and a very short 2A (if there is a 2A peptide at all). Remarkably, other sapeloviruses possess a long 2A and a short L. Notable differences in the organization of the L and 2A proteins can occur among representatives of the same genus, such as in cardioviruses and parecho viruses. Well-defined amino acid motifs are indicated. See main text for details about the H-NC and AIG1 domains. L*, alternative leader protein encoded by an alternative reading frame beginning in the L-encoding sequence; P4, the NPG(P) motif, which interrupts translation at the proline residue; Pro, protease; VPg, primer for RNA synthesis; Zn, zinc finger.

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| Picornaviruses | L | 2A |
|---------------|---|---|
| Cosaviruses   |   |   |
| Senecaviruses | Pro |   |
| Teschoviruses |   | Pro |
| Aphthoviruses |   | Pro |
| Erboviruses   |   |   |
| Cardioviruses |   |   |
| Seal picovirus |   |   |
| Avhepatoviruses |   |   |
| Parechoviruses |   |   |
| Tremoviruses  |   |   |
| Kobuviruses   |   |   |
| Klasseviruses |   |   |
| Sapeloviruses |   |   |
| Enteroviruses |   |   |
| Hepatoviruses |   |   |

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Spliceosome
A complex that is formed of several small nuclear ribonucleoproteins and is involved in splicing.
Table 1 | Properties of the leader proteins of picornaviruses*

| Genus       | Protein | Size (residues) | Isoelectric point | Identifiable domains | Number of species | Interspecies sequence conservation |
|-------------|---------|----------------|------------------|----------------------|------------------|-----------------------------------|
| Senecavirus | SVV L   | 79             | 4.74             | None                 | 1                | NA                                |
|             | TTV L   | 86             | 7.67             | None                 | 1                | NA                                |
|             | FMDV Lab| 199–204        | 4.91–5.13        | Peptidase_C28        | 3                | 32% (51%)                         |
|             | FMDV Lb | 170–175        | 4.50–4.71        |                      |                  | NA                                |
|             | ERAV Lab| 209            | 8.74             |                      |                  | NA                                |
|             | ERAV Lb | 188            | 8.29             |                      |                  | NA                                |
|             | BRV Lab | 207            | 4.86             |                      |                  | NA                                |
|             | BRV Lb  | 183            | 4.37             |                      |                  | NA                                |
| Erbovirus   | ERBV L  | 218–219        | 5.70             | Peptidase_C28        | 1                | NA                                |
| Cardiovirus | EMCV L  | 67             | 3.80             | Zn finger            | 2                | 47% (59%)                         |
|             | TMEV L  | 76             | 3.64             |                      |                  | NA                                |
|             | TMEV L* | 156            | 10.39            |                      |                  | NA                                |
| Sapelovirus | ASV L1  | 215            | 8.66             | None                 | 3                | ND for all 3 species; 15% (34%) for SSV and PSV |
|             | ASV L2  | 236            | 8.54             |                      |                  | NA                                |
|             | SSV     | 88             | 8.95             |                      |                  | NA                                |
|             | PSV     | 84             | 8.46             |                      |                  | NA                                |
| Kobuvirus   | AIV L   | 170            | 5.92             | None                 | 3                | 25% (35%)                         |
|             | BKV L   | 187            | 5.51             |                      |                  | NA                                |
|             | PKV L   | 195            | 5.38             |                      |                  | NA                                |
| Klassevirus | HKV L   | 111–114        | 10.35            | None                 | 1                | NA                                |

AIV, Aichi virus; ASV, avian sapelovirus; BKV, bovine kobuvirus; BRV, bovine rhinovirus B virus; EMCV, encephalomyocarditis virus; ERAV, equine rhinitis A virus; ERBV, equine rhinitis B virus; FMDV, foot-and-mouth disease virus; HKV, human klassevirus; NA, not applicable; ND, not determined; PSV, porcine sapelovirus; PTV, porcine teschovirus; SSV, simian sapelovirus; SV, Seneca Valley virus; TMEV, Theiler’s murine encephalomyelitis virus. *For aphthoviruses, there are two leader (L) proteins, Lab and Lb. For avian sapelovirus, we propose the existence of two leader proteins, L1 and L2 (see Supplementary information S1 (figure)), rather than one L protein. For TMEV, an alternative L protein, L*, is encoded in an alternative reading frame that starts within the L coding sequence.

Amino acid identity (and similarity, in parenthesis) was calculated with the aid of CLUSTAL_X2 alignments, using utilities implemented in BioEdit and the BLOSUM62 similarity matrix.‡ To focus on the core protein sequences, the internal insertions of >15 amino acid residues, as well as terminal insertions, were not taken into account. The peptidase_C28 motif was revealed by BLAST searches in the NCBI Conserved Domain Database.§ For the viral genera with more than one species, the level of interspecies sequence conservation is the suppression of nuclear export of mRNAs, ribosomal RNAs and U spliceosomal small nuclear RNAs. Passive nucleocytoplasmic diffusion of proteins is also facilitated by the cardiovirus L protein, and this effect seems to be caused by L-triggered phosphorylation of nucleoporins.

Effects on innate immunity and viral pathogenicity

One major consequence of the effects of security proteins on host metabolism is the downregulation of innate immunity. FMDV 2A<sup>sm</sup> suppresses interferon production and action<sup>66,67</sup>, largely through the inhibition of nuclear factor-KB-dependent transcription that is caused by the degradation of the p65 subunit of this transcription factor.<sup>55,56</sup> The transcription of genes encoding various cytokines and chemokines (including tumour necrosis factor (TNF; also known as TNFa), T cell-specific protein RANTES (also known as CCL5), myxovirus resistance protein 1 and interferon regulatory factor 7) is also suppressed by FMDV 2A<sup>sm</sup> (REF. 56).

Poliovirus reproduction, which is largely resistant to the effects of interferon, becomes interferon sensitive in 2A<sup>sm</sup> mutants. Introduction of the poliovirus 2A<sup>sm</sup> gene into interferon-sensitive EMCV facilitated its replication in cells pretreated with interferon.<sup>68</sup> The ability of rhinovirus 2A<sup>sm</sup> to cleave mitochondrial antiviral-signalling protein (MAVS; also known as VISA, CARDIF and IPS1), an intermediate in the interferon generation pathway, might contribute to the insensitivity of these viruses to interferons.<sup>69</sup> 2A<sup>sm</sup> also cleaves the catalytic subunit of DNA-dependent protein kinase, which, among other activities, is involved in the induction of pro-inflammatory cytokines.<sup>70</sup> Cardiovirus L also suppresses interferon production by affecting the activation of interferon regulatory factor 3, but the exact mechanism of this interference has not yet been identified.<sup>71,72,73,74</sup> TMEV L* was proposed to suppress the antiviral cytoprotective T cell response in TMEV-infected mice.<sup>75,76</sup>

In line with these observations, the functions of security proteins are less crucial for viral ‘well-being’ in hosts with innate immunity defects. Mutations in cardiovirus L<sup>18,27,28</sup> or 2A<sup>39</sup> or in FMDV L<sup>56</sup> (REF. 56) decrease viral...
conflicting results have been reported on the relation between the security proteins and the apoptotic machinery. For the L*-expressing strains, functional L* is important for the ability of the virus to infect macrophages or microglia in the central nervous system than in wild-type mice. For the L*-expressing strains, functional L* is important for the ability of the virus to infect macrophages or microglia.

One of the components of the innate immune response is apoptosis, which can potentially limit viral reproduction and spread, although certain viruses can subvert the apoptotic machinery to their benefit. Conflicting results have been reported on the relationship between the security proteins and the apoptotic machinery. Ectopic expression of enterovirus 2A triggers an apoptotic response and enhances the sensitivity of cells to the apoptogenic activity of tumour necrosis factor. However, in the context of the whole genome, 2A possesses anti-apoptotic activity. Similarly, expression of TMEV L in macrophage-like cells induces apoptosis, as occurs during TMEV infection of these cells, whereas EMCV and mengovirus L are anti-apoptotic in the context of the whole virus (at least in HeLa cells, in which the virus itself elicits necrotic death). Whether this apparent discrepancy is a result of the use of different assays or hosts or of the intrinsic peculiarities of cardiovirus L proteins remains unknown. TMEV L* has also been reported to exhibit anti-apoptotic activity.

Several illuminating examples strongly suggest that security proteins can markedly modulate viral pathogenicity. FMDV lacking L is highly attenuated,

### Table 2: Properties of the 2A proteins and peptides of picornaviruses

| Virus Genus | Species | Size (residues) | Isoelectric point | Identifiable domains/motifs | Number of species | Inter-species sequence conservation |
|-------------|---------|-----------------|-------------------|-------------------------------|------------------|------------------------------------|
| Cosavirus   | All 5 species | 30–37           | ND                | NPG(P)                        | 5                | ND                                 |
| Senecavirus | SVV     | 9               | ND                | NPG(P)                        | 1                | NA                                 |
| Teschoirus  | PTV     | 21              | ND                | NPG(P)                        | 1                | NA                                 |
| Erboirus    | ERBV    | 16              | ND                | NPG(P)                        | 1                | NA                                 |
| Aphthovirus | FMDV    | 18              | ND                | NPG(P)                        | 3                | ND                                 |
|             | ERAV    | 18              | ND                |                               |                  |                                    |
|             | BRV     | 19              | ND                |                               |                  |                                    |
| Cardiovirus | EMCV    | 143             | 9.67              | NPG(P)                        | 2                | 24% (42%)                          |
|             | TMEV    | 133             | 8.89              |                               |                  |                                    |
| Sapelovirus | ASV     | 12              | ND                | None                          | 3                | ND for all 3 species; 27% (39%) for SSV and PSV |
|             | SSV     | 292–302         | 5.00              | Protease§                      |                  |                                    |
|             | PSV     | 226             | 5.55              | Protease§                      |                  |                                    |
| Kobuvirus   | AiV     | 111             | 5.92              | H-NC¶                         | 3                | 54% (66%)                          |
|             | BKV     | 134             | 5.51              |                               |                  |                                    |
|             | PKV     | 136             | 5.38              |                               |                  |                                    |
| Parechovirus| LV 2A1  | 20              | ND                | NPG(P)                        | 2                | 44% (64%) for 2A2 of LV and 2A of HPeV |
|             | LV 2A2  | 135             | 6.60              | H-NC                          |                  |                                    |
|             | HPeV    | 160             | 5.28              | H-NC                          |                  |                                    |
| Avihepatovirus | DHV 2A1 | 20              | ND                | NPG(P)                        | 1                | NA                                 |
|             | DHV 2A2+2A3 | 285         | 8.65              | AIG1 and H-NC                 |                  |                                    |
| Hepatovirus | HAV     | 189             | 8.78              | None                          | 1                | NA                                 |
| Tremovirus  | AEV     | 163             | 8.22              | H-NC                          | 1                | NA                                 |
| Enterovirus | All 10 species | 142–150 | 5.22–6.30 | Pico_P2A                      | 10               | 35% (51%)                          |
| Klassevirus | HKV     | 126             | 4.80              | None                          | 1                | NA                                 |
| Unclassified | SePV-1 2A1 | 29               | ND                | NPG(P)                        | 1                | NA                                 |
|             | SePV-1 2A2 | 100             | 7.92              | None                          | 1                | NA                                 |

AEV, avian encephalomyelitis virus; AiV, Aichi virus; ASV, avian sapelovirus; BKV, bovine kobuvirus; BRV, bovine rhinitis B virus; DHV, duck hepatitis A virus; EMCV, encephalomyocarditis virus; ERAV, equine rhinitis A virus; ERBV, equine rhinitis B virus; FMDV, foot-and-mouth disease virus; HAV, hepatitis A virus; HKV, human hepatitis A virus; HPeV, human parechovirus; LV, L-jungan virus; NA, not applicable; ND, not determined; PKV, porcine kobuvirus; PSV, porcine sapelovirus; PTV, porcine teschovirus; SePV-1, seal picornavirus type 1; SSV, simian sapelovirus; SVV, Seneca Valley virus; TMEV, Theiler’s murine encephalomyelitis virus. *For those viruses with more than one 2A protein, the specific protein analysed is indicated. †According to the data in GenBank. ‡Values were calculated only for peptides with >40 residues using ProtParam. §For the viral genera with more than one species, the level of interspecies amino acid identity (and similarity, in parentheses) was calculated with the aid of CLUSTAL_X2 alignments, using utilities implemented in BioEdit and the BLOSUM62 similarity matrix. ¶To focus on the core protein sequences, the internal insertions of >15 amino acid residues, as well as terminal insertions, were not taken into account. *These are manually recognizable motifs. †One of the strains is reported to harbour an NPR(P) motif instead of NPG(P).
and both L\textsuperscript{23,90} and L* (REFS 33, 81, 88) of TMEV strains BeAn 8386 and DA have been implicated in persistence in the central nervous system and in demyelinating disease. 2A\textsuperscript{Pro} of human coxsackievirus B4, which can cleave dystrophin in the cardiac muscle, seems to be involved in the pathogenesis of human acquired dilated cardiomyopathy\textsuperscript{89}. Moreover, a major virulence determinant of swine vesicular disease virus, an enterovirus, has been mapped to 2A\textsuperscript{Pro} (REF 91).

**Exchangeability and dispensability**

Notwithstanding the low level of conservation of L proteins between EMCV and TMEV (TABLE 1), these proteins can be functionally exchanged with respect to their ability to inhibit interferon formation and to ‘open’ nuclear pores\textsuperscript{90}. The replacement of L in the full-length cardiovirus genome with FMDV L\textsuperscript{Pro} generates a virus that can overcome host defences more efficiently than its leaderless counterpart, although it has lower fitness\textsuperscript{92,93}. Such interchangeability of structurally and biochemically distinct proteins attests to the similarity of their biological functions.

It is hardly by chance that viruses that encode long or multiple Ls tend to have a short 2A or lack 2A altogether (assuming that 2A\textsuperscript{a} is not a security protein), and vice versa (FIG. 1). For example, simian and porcine sapeloviruses harbour a long 2A and a short L, whereas avian sapelovirus encodes the longest L identified to date and the predicted 2A ORF is very short, if it encodes a protein at all. This tendency is consistent with the notion that L and 2A have similar roles in virus–host interactions. As already mentioned, some picornaviruses, for example, coxsaviruses, encode no security proteins. Even viruses that do encode security proteins can, under certain circumstances, survive and replicate after these proteins have been inactivated or eliminated. Notably, cardiovirus L\textsuperscript{31,48,77,78} and L* (REFS 34, 81, 94) and FMDV L\textsuperscript{Pro} (REF 95) are not essential for viral viability, and extended deletions in 2A of cardioviruses\textsuperscript{42,49} and hepatitis A virus\textsuperscript{96,97} do not kill these viruses. Furthermore, although some data suggest that poliovirus 2A\textsuperscript{Pro} has an essential replicative function\textsuperscript{98}, recent experiments have demonstrated its dispensability for viral viability\textsuperscript{99}.

**Division of labour in picornavirus proteins**

Although this Review focuses on the counter-defensive functions of security proteins, it should be kept in mind that other picornavirus proteins are also often engaged in similar functions. The targets of 3C\textsuperscript{Pro} (or its proteolytically active precursors) might include proteins that are involved in innate immunity\textsuperscript{99–102}. 2B and 3A are also important players in the virus–host struggle, being involved in the rearrangement of cytoplasmic membranes and in suppression of trafficking, secretion and antigen presentation on cellular plasma membranes\textsuperscript{84,103–105}. 2C can also participate in some of these activities\textsuperscript{103}. Even specialized proteins such as VPg\textsuperscript{106} and capsid proteins\textsuperscript{107} can sometimes assist in overcoming host defences. In all these cases, however, the ‘security’ functions are neither the main nor the conserved roles of these proteins.

Conversely, as well as representing a dedicated counter-defensive system, security proteins can be directly involved in viral reproduction. The products of the cleavage of eIF4G, and possibly of some other host proteins, by FMDV L\textsuperscript{Pro} and enterovirus 2A\textsuperscript{Pro} can stimulate IRES-dependent cell-free translation\textsuperscript{108,109}, the effect of 2A\textsuperscript{Pro} is partly the result of stabilization of the viral RNA\textsuperscript{110}. The physiological relevance of these phenomena is unclear, however. The poliovirus IRES dysfunction that is caused by some mutations can be compensated for by mutations in 2A in a host-dependent manner\textsuperscript{111}, suggesting the participation of host proteins. Cardiovirus L was also implicated in control of IRES activity, because EMCV RNA with deletions in the L-coding sequence exhibited a decrease in cell-free translatability\textsuperscript{112}. Poliovirus 2A\textsuperscript{Pro} was reported to stimulate strand initiation of negative-strand RNA, and this was seemingly independent of its effects on RNA stability and translation\textsuperscript{113}. Deletion of a carboxy-terminal sequence from this protein does not substantially affect its protease activity but does inhibit RNA replication\textsuperscript{112}. Deletion of the amino-terminal region notably, but incompletely, suppresses replication of the relevant replicon\textsuperscript{114}. Similarly, deletions in 2A of Aichi virus (a kobuvirus) inhibit viral RNA replication\textsuperscript{115}. However, the mechanisms responsible for these effects have not been elucidated, casting doubts on whether these 2A proteins affect viral replication directly or by modulating host cell activities.

A role for hepatitis A virus 2A in virion assembly and maturation has been established\textsuperscript{97,115}. The primary co-translational cleavage of the viral polyprotein takes place between 2A and 2B and is accomplished by the viral proteinase 3C\textsuperscript{Pro} (or its precursor), leaving the 2A sequence fused to the carboxyl terminus of capsid protein VP1 (REFS 116, 117). In immature virions, VP1 retains this 2A extension, which is eventually cleaved off by an unidentified enzyme\textsuperscript{116}. The involvement of 2A in the maturation of some other picornaviruses cannot be excluded therefore. Notably, the primary co-translational scission of the cardiovirus polyprotein generates a fusion between 2A and the capsid protein precursor\textsuperscript{25}.

**Origins and evolution of security proteins**

Obviously, it is not possible to construct a genealogical tree of either L or 2A, because they are unrelated. These proteins are not considered at all in the hypothesis on the origin of picorna-like viruses\textsuperscript{1}, and only the short, translation-interrupting 2A peptides (that is, the 2A\textsuperscript{a} peptides) are briefly mentioned in the proposal on picornavirus classification\textsuperscript{119}. There is no correlation between the nature (or even the presence) of security proteins and either the type of IRES that lies upstream of the L protein or the RdRP lineage (which is generally accepted as the most reliable indicator of viral relatedness) (FIG. 2).

The diversity of the security proteins and their absence from many picornaviruses suggest that they are independent and late evolutionary acquisitions\textsuperscript{8,120}. It can be speculated that the most ancient of the 2A molecules are the 2A\textsuperscript{a} peptides, as hinted by their presence in most picornavirus genera, including those belonging to different lineages (FIG. 2). Interruption of polyprotein synthesis
The RdRP tree was constructed by using sequences were taken from type 2 (HPeV 2A proteins that contain the NPG(P) motif (which interrupts translation at the indicated proline residue; shown by P) viruses (for example, it is present in tremoviruses but absent in hepatoviruses). The same pattern is characteristic of the avihepatoviruses, parechoviruses and tremoviruses on the other), but this motif is not shared by more closely related 2A proteins with the H-NC containing regions of the 2A protein. Well-defined amino acid motifs are indicated. Viral RdRP protein sequences were constructed using CLUSTALW with default parameters. Solenopsis invicta virus 2 (SolV-2) was used as the outgroup. AEV, avian encephalomyelitis virus; AIV, Aichi virus; ASV, avian sapelovirus; EMCV, encephalomyocarditis virus; ERBV, equine rhinitis B virus; FMDV, foot-and-mouth disease virus; HAV, hepatitis A virus; HCoSV-A, human cosavirus A; HPV-1, human poliovirus type 1; Mahoney; HRV, human rhinovirus A101; PKV, porcine kobuvirus; Pro, protease; PSV, porcine sapelovirus; PTV, porcine teschovirus; SalV, Salivirus NG-A; SSV, simian sapelovirus; SVV, Seneca Valley virus; TMEV, Theiler’s murine encephalomyelitis virus; Zn, zinc finger.

**Figure 2** | Relationships between the presence of distinct security proteins and other evolutionary hallmarks of picornaviruses. The distribution of security proteins among different viruses is not congruent with either the type of internal ribosome entry site (IRES); the key cis-acting element responsible for cap-independent translation of picornavirus RNAs or the topology of the RNA-dependent RNA polymerase (RdRP) tree. For example, viruses harbouring type II IRESs can possess different L proteins (aphthoviruses and cardioviruses) or be devoid of this protein (cosaviruses). A similar situation occurs with viruses that use type IV IRESs. Conversely, different kobuviruses can possess unrelated IRESs. 2A proteins with the H-NC motif are present in viruses of distant RdRP lineages (kobuviruses on the one hand and aviparvoviruses, parechoviruses and tremoviruses on the other), but this motif is not shared by more closely related viruses (for example, it is present in tremoviruses but absent in hepatoviruses). The same pattern is characteristic of the 2A proteins that contain the NPG(P) motif (which interrupts translation at the indicated proline residue; shown by P). The closely related seal picornavirus type 1 (SePV-1), duck hepatitis A virus (DHV), Ljungan virus (LV) and human parechovirus type 2 (HPeV-2) each harbour a distinct 2A protein. Well-defined amino acid motifs are indicated. Viral RdRP protein sequences were taken from GenBank. Multiple alignments of protein sequences were constructed using CLUSTAL-X2. The RdRP tree was constructed by using MrBayes with default parameters. Solenopsis invicta virus 2 (SolV-2) was used as the outgroup. AEV, avian encephalomyelitis virus; AIV, Aichi virus; ASV, avian sapelovirus; EMCV, encephalomyocarditis virus; ERBV, equine rhinitis B virus; FMDV, foot-and-mouth disease virus; HAV, hepatitis A virus; HCoSV-A, human cosavirus A; HPV-1, human poliovirus type 1; Mahoney; HRV, human rhinovirus A101; PKV, porcine kobuvirus; Pro, protease; PSV, porcine sapelovirus; PTV, porcine teschovirus; SalV, Salivirus NG-A; SSV, simian sapelovirus; SVV, Seneca Valley virus; TMEV, Theiler’s murine encephalomyelitis virus; Zn, zinc finger.

After translation of the capsid proteins might be advantageous for viral reproduction. It might, for example, facilitate proper protein folding, ensure optimal kinetics of protein synthesis or control the ratios of structural to non-structural proteins, which could theoretically be achieved by incomplete translational re-initiation at the second proline residue of the NPG(P) motif. It is worth noting that there is a difference in the translation factor requirements for translation of the EMCV polyprotein upstream and downstream of the 2A–2B boundary.

Strikingly, 2A proteins — or more accurately, the DXE XVPG(P) motifs (where X is any amino acid) that are characteristic of these peptides — are found in some other picorna-like and unrelated viruses, where they seem to serve the same function. Assuming that the acquisition of NPG(P) was an early event, this motif might have been lost by some parechoviruses (and other picornaviruses) at a certain step of evolution. In contrast to picornaviruses, the acquisition of 2A proteins by members of some other families of RNA viruses has been proposed to have occurred at a late stage. The idea that these peptides might have originated in picornaviruses is an attractive hypothesis. The NPG(P) motif can also be found in several cellular proteins, but the current data do not allow researchers to determine whether the recoding ability of this motif was a viral or cellular invention.

The non-NPG(P)-containing regions of the 2A proteins are unrelated acquisitions. Cardiovirus 2A possesses these additional moieties in its amino-terminal region, whereas other viruses of this subset (parechoviruses,
Inhibition of cellular transcription (proteolysis of translation factors)

Inhibition of the interferon system (interference with IRF3 activation)

Inhibition of cellular translation (inhibition of nuclear mRNA export)

Inhibition of cellular transcription (predominantly of cytokine genes)

Inhibition of the interferon system (proteolysis of the p65 subunit of NF-xB)

Inhibition of cellular translation (proteolysis of translation factors)

Inhibition of cellular translation (proteolysis of translation factors)

Inhibition of cellular transcription (proteolysis of translation factors)

Control of the apoptotic machinery

Permeabilization of the nuclear envelope

Inhibition of active nucleocytoplasmic transport

Entervirus 2A

Cardiovirus L

Aphthovirus L*

Figure 3 | Major biological functions of the best studied but unrelated security proteins. There is a striking similarity between the functional activities of the enterovirus 2A protease (2A^m) and the cardiovirus leader protein (L), but the underlying mechanisms by which these functions are carried out are fundamentally different. By contrast, the known functional activities of aphthovirus L, which is a protease (L^m), seem to be more limited. IRF3, interferon regulatory factor 3; NF-xB, nuclear factor-xB.

Avihepatoviruses and seal picornavirus type 1) contain these moieties in their carboxy-terminal regions (Fig. 1). This may suggest that these moieties were acquired after the NPG(P) motif. In avihepatoviruses and some parechoviruses, these newly acquired sequences harbour the H-NC motif, which is also present in the NPG(P)-lacking 2A proteins of other parechoviruses, kobuviruses and treomaviruses (that is, viruses of different RdRP lineages) (Fig. 2). A plausible hypothesis is that H-NC-containing proteins from cellular organisms were hijacked by certain picornaviruses on several independent occasions, but the possibility that 2A^p peptides were formed by deletions from larger 2A proteins cannot be ruled out.

Taking into account the chymotrypsin-like fold that is found in enterovirus 2A^m, it is reasonable to assume that this protein was derived from picornavirus 3C^m or a cellular protease. A similar assumption could perhaps be made for the putative sapelovirus 2A protease. There are no obvious clues to the possible origins of the 2A proteins of hepatoviruses and kashaviruses or of the non-NPG(P) moieties of the 2A proteins of cardioviruses and seal picornavirus type 1.

With regard to the picornavirus L proteins, one hypothesis is that the papain-like L^m proteases of aphthoviruses and erboviruses originated from cellular enzymes. No obvious relatives of other L proteins can currently be identified among cellular or viral proteins. Only the origin of cardiovirus L* seems certain: the first L* probably came into being accidentally, by translation of an alternative reading frame, and was then shaped by mutations preserving the functional integrity of L, VP4 and VP2. It is worth noting that recently identified human TMEV-like cardioviruses lack this alternative reading frame. Theoretically, several mechanisms could underlie the acquisition of security proteins. For example, one possibility is that viral genes were duplicated and subsequently substantially modified (such a scenario can be imagined for the origin of enterovirus 2A^m (Ref. 125)). Other scenarios are: recombination with viral or cellular RNAs encoding related proteins such as proteases; conversion of non-coding RNA sequences into coding sequences (which could occur through different mechanisms, such as interspecies recombination (Ref. 126) (V.I.A., A.P.G., E. V. Khitrina and W. J. Melchers, unpublished observations), or introduction or activation of an upstream in-frame AUG codon; frame shifting (Ref. 128) (or double frame shifting, in the case of 2A proteins); and using an alternative reading frame. Unfortunately, we can only pinpoint a definite mechanism for the case of L*, for which the last mechanism has obviously been operative.

The acquisition of security proteins, by whatever mechanism, required that the new ‘additions’ did not interfere with the function of the adjacent viral proteins — VP4 (or VP0) in the case of L, and VP1 and VP2 in the case of 2A. The new proteins should either be separated from these neighbours (by self-proteolysis, the action of other viral proteases or translation interruption) or, if they remain fused, they should not impair the functions of these neighbours (as is the case with the hepatitis A virus VP1–2A fusion).

A separate issue is the evolution of the security proteins themselves. It was proposed that the L proteins of TMEV and EMCV diverged during evolution to adapt to the different replication fitnesses of these viruses. The data are too scarce, however, for any generalizations at this point.

Conclusions

L and 2A constitute a distinct and remarkable set of picornavirus proteins. They exhibit striking structural and biochemical diversity but (with the exception of the 2A^p peptides) accomplish similar biological functions by countering host defensive reactions. However, their abilities to solve similar problems might involve fundamentally different molecular mechanisms. This is illustrated in Fig. 3, which summarizes the properties of the three best studied security proteins. The biological functions of enterovirus 2A^m and cardiovirus L are strikingly similar: inhibition of host macromolecular synthesis, permeabilization of the nuclear envelope and inhibition of active nucleocytoplasmic transport, suppression of specific innate immunity mechanisms and control of the apoptotic machinery of the host cells. No less striking is the difference in the mechanisms by which the two proteins achieve these goals. Conversely, aphthovirus L^m can solve only a subset of these problems. An intriguing
question is how, and whether, the diverse security func-
tions exhibited by a given protein (Fig. 3) are related to
each other. The fact that mutations inactivating one
function usually also impair other functions suggests
the existence of a common upstream target.

Most of the picornavirus L and 2A proteins still await
researchers’ attention. Nevertheless, the data discussed
here allow us to provisionally assign security functions
even to those L and 2A proteins that have not yet been
characterized. Indeed, L and 2A do not definitely belong
to the set of essential reproductive proteins that ensure
translation and replication of the viral genome (although
2A of hepatitis A virus assists virion maturation).

We propose that the concept of security proteins is of
general relevance and can be applied to viruses other than
picornaviruses. The hallmarks of these proteins are as fol-
loows: structural and biochemical unrelatedness or even
absence in related viruses; the dispensability of the entire
protein or its functional domains for viral viability; and,
for mutated versions of the proteins, fewer detrimental
effects on viral reproduction in immune-compromised
hosts than in immune-competent hosts. Possessing one
of these features would make a viral protein a good can-
didate security protein, whereas a combination of these
features would probably confirm this designation.

Viruses with large DNA genomes possess impres-
sive arsenals of security proteins. The complement of
security proteins is much more limited in RNA viruses
but is sufficient for their evolutionary success. There
are also tentative examples of security proteins in
non-picornavirus RNA viruses. Coronaviruses have
several so-called accessory proteins, which are neither
conserved nor essential and exhibit the capacity to sup-
press host innate immunity by a range of mechanisms124.

The interaction between host defences and viral counter-
defence is certainly one of the key factors underlying the
pathogenicity of picornaviruses and other viruses. This
interaction cannot be fully understood without elucida-
tion of the roles of the security proteins. Treatment and
prevention of viral diseases may also markedly benefit
from such elucidation. The study of security proteins is
therefore an underdeveloped but highly promising
research area.

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