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MINIREVIEW

Structure, function and evolution of the hemagglutinin-esterase proteins of corona- and toroviruses

Raoul J. de Groot

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Abstract Virus attachment to host cells is mediated by dedicated virion proteins, which specifically recognize one or, at most, a limited number of cell surface molecules. Receptor binding often involves protein-protein interactions, but carbohydrates may serve as receptor determinants as well. In fact, many different viruses use members of the sialic acid family either as their main receptor or as an initial attachment factor. Sialic acids (Sias) are 9-carbon negatively-charged monosaccharides commonly occurring as terminal residues of glycoconjugates. They come in a large variety and are differentially expressed in cells and tissues. By targeting specific Sia subtypes, viruses achieve host cell selectivity, but only to a certain extent. The Sia of choice might still be abundantly present on non-cell associated molecules, on non-target cells (including cells already infected) and even on virus particles themselves. This poses a hazard, as high-affinity virion binding to any of such "false" receptors would result in loss of infectivity. Some enveloped RNA viruses deal with this problem by encoding virion-associated receptor-destroying enzymes (RDEs). These enzymes make the attachment to Sia reversible, thus providing the virus with an escape ticket. RDEs occur in two types: neuraminidases and sialate-*O*-acetylesterases. The latter, originally discovered in influenza C virus, are also found in certain nidoviruses, namely in group 2 coronaviruses and in toroviruses, as well as in infectious salmon anemia virus, an orthomyxovirus of teleosts. Here, the structure, function and evolution of viral sialate-*O*-acetylesterases is reviewed with main focus on the hemagglutinin-esterases of nidoviruses.

R.J.d. Groot (✉)
Virology Section, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
e-mail: R.Groot@vet.uu.nl
Tel: +31-30-2531463
Fax: +31-30-2536723

Introduction

For viruses, to successfully initiate infection, it is vital to attach to appropriate host cells. Selectivity is ensured by receptor-binding proteins on the virion, which are tailored to recognize and bind to specific cell surface molecules, *i.e.* glycoproteins and glycolipids. Virion attachment often relies on protein-protein interactions, but carbohydrates may serve as (co-)receptor-determinants as well. Prominent among the latter are the sialic acids (Sias), a diverse family of 9-carbon negatively-charged monosaccharides, which commonly occur as terminal residues of glycoconjugates (for reviews, see [1–3]). Sias exist in a large variety, which arises from differential modification of the parental molecule 5-*N*-acetylneuraminic acid (Neu5Ac). One of the most common types of modifications is *O*-acetylation, which may occur at C4, C7, C8, and C9. Further adding to Sia diversity are differences in glycosidic linkage (α2-3, α2-6, α2-8 or α2-9) to the penultimate residue of the sugar chain. By targeting one particular type of Sia, viruses achieve tissue- and cell specificity, at least to a certain extent. These Sias might still abound on soluble molecules in extracellular fluids, on the surface of non-permissive cells, and in the mucus, which protect the epithelia of the respiratory and digestive tracts. Conceivably, the binding of virions to such "decoy" receptors would result in loss of infectivity. Moreover, upon completion of the replicative cycle, loss of infectivity might result from aggregation of virus progeny at the surface of the infected host cell or from Sia-mediated virus self-aggregation. Some enveloped RNA viruses evade these perils by encoding virion-associated receptor-destroying enzymes (RDEs). These viral enzymes, which fall into two classes -the neuraminidases

(sialidases) and the sialate-*O*-acetylesterases-, provide an escape ticket allowing high-affinity yet reversible binding to Sia receptor determinants. Below, I will first briefly dwell on early studies, which led to the discovery of viral RDEs, and then discuss the structure, function and evolution of viral sialate-*O*-acetylesterases with particular focus on the hemagglutinin-esterase proteins of corona- and toroviruses. For additional information on the RDEs of influenza C and coronaviruses, the reader is referred to excellent reviews by Herrler and Klenk [4] and Brian *et al*. [5].

Early studies: the discovery of virion-associated receptor-destroying enzymes

In the early 1940's, George Hirst [6,7] described the specific adherence of influenza A and B viruses to chicken erythrocytes. The viruses induced clumping of the red blood cells (hemagglutination), apparently resulting from multivalent binding of viral factors (the "hemagglutinins") to specific receptors on the cell surface. Hirst realized that these receptors might be the same as those mediating viral attachment to natural host cells. He also noted that influenza viruses bound to the erythrocytes only to elute from them again in a time- and temperature-dependent fashion. In the process, the erythrocytes became refractory to hemagglutination as the receptors at the cell surface were apparently lost. He concluded that the virus preparations hence must possess both receptor-binding and receptor-destroying enzyme (RDE) activities [6]. Interestingly, this phenomenon was not unique to influenza viruses: shortly after Hirst's landmark discoveries, similar observations were reported for the paramyxoviruses Newcastle disease (NDV) and mumps virus.

Treatment of chicken erythrocytes with concentrated preparations of influenza A/PR/8 virus or influenza B/Lee/40 virus resulted in loss of the receptors for either strain [6]. Moreover, the RDEs of the influenza viruses also destroyed the paramyxovirus receptors and *vice versa* [8]. Evidently, all these viruses recognized a common receptor determinant, which, in an impressive biochemical effort, was identified as 5-*N*-acetylneuraminic acid [9]. The evidence was based upon the observation that influenza B/Lee/40 virus enzymatically cleaved off this compound from human urine mucin -a strong influenza virus inhibitor, which had been isolated from 10 L of "männlichen Sammelurins" [9]. These studies defined one main type of viral receptor-destroying enzymes (RDEs), the neuraminidases [10].

Already by then, there were indications for the existence of another class of viral RDEs. Hirst [11] had found that a particular human respiratory virus isolate "1233" -in fact an influenza C virus strain- resembled the members of the so-called "mumps-NDV-influenza" (MNI) group in that it bound to and destroyed receptor determinants on chicken erythrocytes. Yet, while strain 1233 destroyed its own receptors, it left those of the "MNI group" unaffected. In turn, the RDEs of the latter myxoviruses failed to destroy the receptors for strain 1233 [11]. These receptors, however, were sensitive to degradation by bacterial neuraminidases [12,13], indicating that sialic acid must be an essential component. To reconcile these paradoxical findings, Herrler *et al.* [12] suggested that there might be additional requirements for sialic acid in order to act as a receptor for influenza C virus, one of which mentioned was "*modification of the sialic acid, e.g. O-acetylation*". To accommodate previous reports, which had found influenza C virus preparations to be devoid of neuraminidase activity [14,15], the authors offered the possibility that the RDE might actually be "*an esterase that deacylates neuraminic acid*". Indeed, shortly thereafter, the same team of researchers provided biochemical evidence that influenza C virus preparations contain sialate-*O*-acetylesterase activity [16]. RDE-mediated inactivation of the bovine submaxilary mucin (BSM) and rat α1-macroglobulin, *i.e.* inhibitors of influenza C virus-induced hemagglutination, correlated with the conversion of their predominant Sia constituent, 5-*N*-acetyl-9-*O*-acetylneuraminic acid ($Neu5,9Ac_2$), to 5-*N*-acetylneuraminic acid (Neu5Ac). The importance of 9-*O*-acetylated sialic acid as a specific primary receptor determinant for influenza C virus was corroborated by studies in which virus-mediated agglutination of neuraminidase-treated asialo-erythrocytes was restored upon resialylation with $Neu5,9Ac_2$, but not with 5-*N*-glycolylneuraminic acid (Neu5Gc) or with Neu5Ac [17]. Perhaps more importantly, it was also demonstrated that (i) pre-treatment with neuraminidase or with sialate-*O*-acetylesterase rendered tissue culture cells resistant to infection with influenza C virus and (ii) that this defect could be restored through resialylation of these cells with $Neu5,9Ac_2$-containing bovine brain gangliosides. In fact, even cell lines resistant to influenza C virus could be made permissive via this strategy, indicating that $Neu5,9Ac_2$ is a major determinant for influenza C virus host cell tropism [18].

These early studies already convey the essence of viral adaptation to Sia receptor usage. Viruses do not indiscriminately bind to "sialic acid". Instead, they have evolved to target specific Sia subtypes. Whether a particular Sia is recognized by a particular virus might depend on the penultimate residue of the sugar chain to which the Sia is linked, the type of glycosidic linkage, and/or the absence or presence of specific Sia modifications. Evidently, for those viruses with RDEs, the substrate preference of the enzyme and the ligand specificity of the receptor-binding protein must match. The difference between the two types of RDEs is explained in Figure 1. Whereas neuraminidases ("sialidases") remove the complete Sia moiety by catalyzing the hydrolysis of the

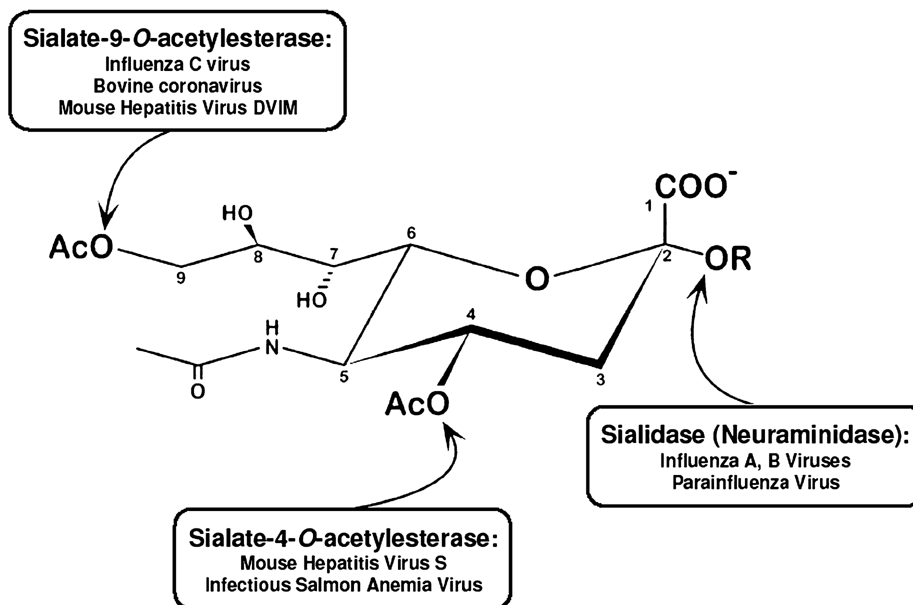


Fig. 1 Substrate specificities of viral sialidases and sialate-*O*-acetylesterases (adapted from Regl *et al.* [97])

α-ketosidic linkage to the adjacent D-galactose or *N*-acetyl-D-galactosamine [10], sialate-*O*-acetylesterases have a more sophisticated mode of action and merely cleave off specific *O*-acetyl groups (Figure 1) [19]. This also implies that for viruses employing the latter type of RDE, recognition of the Sia receptor determinant critically depends on the presence of the *O*-acetyl moiety.

The influenza *C* virus hemagglutinin-esterase-fusion protein: the sialate-*O*-acetylesterase archetype

Virions of influenza A and B viruses are studded with two different types of spikes, the hemagglutinin (HA), which mediates receptor-binding and pH-dependent membrane-fusion and the neuraminidase (NA), the viral RDE [20]. In contrast, influenza C virus possesses only one type of spike, which combines all three functions [21–27] and hence is commonly referred to as the hemagglutinin-esterase-fusion protein (HEF). Like its homologue HA, to which it bears limited yet significant primary sequence similarity [28–31], HEF is a homotrimer of an N-glycosylated type I membrane protein [32–34]. The monomer, HEF_0, is synthesized as an 88 kDa precursor and proteolytically cleaved by a host cell protease to yield a 65 K N-terminal subunit, HEF_1, and a 30 K membrane-anchored C-terminal subunit, HEF_2 [32]. These subunits remain covalently-linked via disulfide-bonding [35]. Again as for HA, cleavage of HEF is essential for fusion activity [25,26].

By affinity labeling with the [^{3}H]labeled esterase inhibitor diisopropylfluoro-phosphate (DFP), the esterase domain was mapped to the N-terminal subunit HEF_1 [22]. DFP specifically binds to Ser^{57} (as numbered from the N-terminus in the mature protein), which was thus identified as the main catalytic active-site residue [36]. Ser^{57} and its adjacent residues form a consensus motif, Phe-Gly-Asp-Ser, typically found in the active site of serine-based esterases and proteases. Vlasak *et al.* [36] therefore suggested that, in analogy, the active site of the HEF is likely comprised of a catalytic triad with Asp and His as the additional key residues. The identities between HEF and other serine hydrolases were too limited, however, to allow unambiguous identification of the latter two residues solely by comparative sequence analysis. With the elucidation of the HEF crystal structure [31,37], this question and many more relating HEF structure and function have now been solved.

Despite an overall primary sequence identity of only 11%, the similarity between the 3D structures of HEF and HA is astounding [31]. The main differences are within the N-terminal subunits, which form the globular top part of the spikes and, which, in the case of HEF, contain the receptor-binding (R) and RDE esterase (E) domains. The C-terminal HEF_2 subunit, together with segments of HEF_1, comprises the fusion domain (F). In the trimer, HEF_2 subunits constitute the elongated membrane-anchored stalk, at the core of which long central α-helices form a triple-stranded interphase (Figure 2a).

Each HEF_1 subunit contains two sites that can accomodate Neu5,9Ac$_2$ (Figure 2a). The actual receptor-binding sites are cavities at the tip of the globular head domain with Tyr^{127}, Thr^{170} and Gly^{172} involved in substrate binding. Interaction with the 9-*O*-acetyl side group, the most critical determinant for receptor recognition, is mediated a.o. by the hydroxyl group of Tyr^{224} and the guanidino group of Arg^{236} which contact the acetyl carbonyl oxygen. Moreover, Phe^{225} and Phe^{293} and Pro^{271} form a nonpolar pocket into which the acetyl methyl group can be fitted (Figure 2a and b) [31].

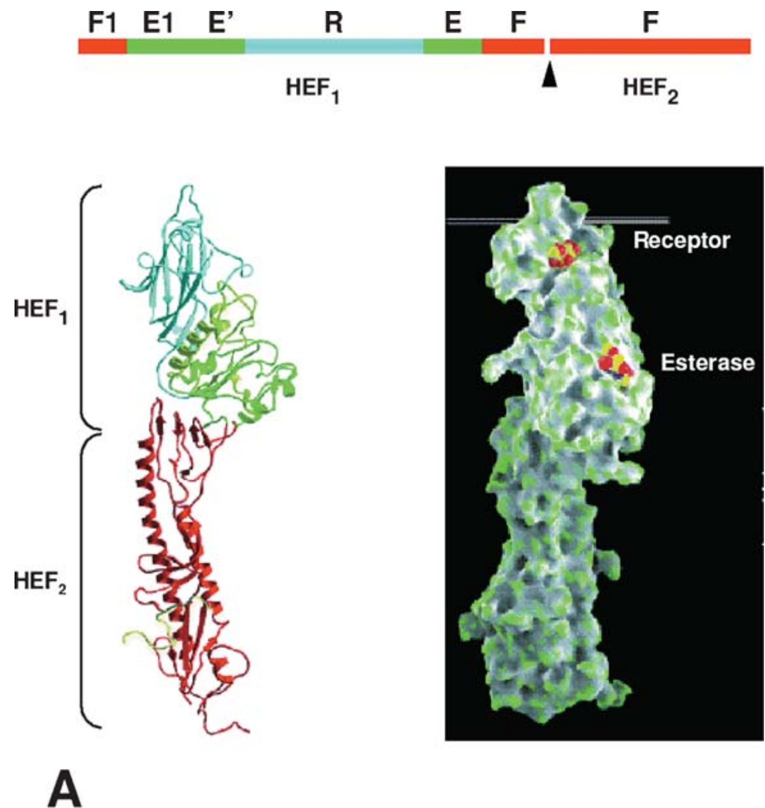


Fig. 2 (a) Structure of the influenza C virus HEF monomer. In the ribbon structure (left), sequence segments are colored by domains (F, red; E, green; R, blue); the fusion peptide is shown in yellow. The monomer surface (right) shows the locations of the receptor binding site and the catalytic site of the esterase. The linear order of the sequence segments in HEF, colored by domain, is shown above. (b) Ligand bound to the receptor-binding site. (c) HEF enzymatic active site with the substrate in yellow and the catalytic triad is shown in green. Reprinted from Rosenthal *et al.* [31] with permission

The importance of this latter site for receptor recognition is underlined by observations that mutations of residues adjacent to Pro^{271} (Asp$^{269}\rightarrow$ Asn, Thr^{270} $\rightarrow$ Leu or Ile and Thr$^{272}\rightarrow$ Ile) are associated with increased binding efficiency to Neu5,9Ac$_2$-containing receptors [38–41].

The enzyme active site, composed of Ser^{57}, His^{355} and Asp^{352}, is located at the widest part of the trimer near the base of the globular head region. The NH group of Gly^{85} and the side chain of Asn^{117} together with the NH group of Ser^{57} form the oxyanion hole, whereas Arg^{322} apparently assists

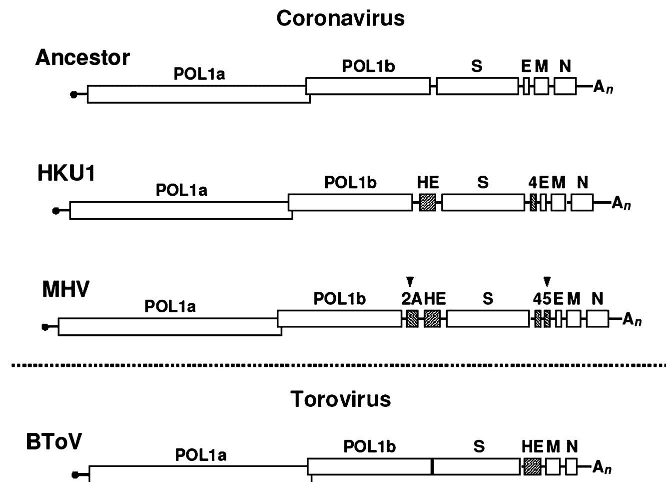


Fig. 3 Schematic representation of the genome organization of group 2a coronaviruses (HCoV-HKU1, MHV and a hypothetical group 2 ancestor; upper panel) and of toroviruses (BToV; bottom). Boxes represent the various genes, with those for the polymerase polyproteins (POL1a, POL1b), and for the 2A, HE, S, E, M, N, ns4 and ns5 proteins indicated; the boxes indicating the accessory genes are marked by hatching. "An" indicates the poly(A) tail, the black dot the cap structure

in substrate binding by donating two hydrogen bonds to the sialoside carboxylate group (Figure 2c) [31,37]. Consistent with their role in catalysis, substitution of Ser^{57} and His^{355} by Ala reduces esterase activity by more than 90% [42].

Enter the nidovirus sialate-*O*-acetylesterases

Shortly after the identification of HEF as a sialate-*O*-acetylesterase [16], it became clear that this type of RDE is not restricted to influenza C virus, but, astonishingly, also occurs in certain coronaviruses [43] as well as in toroviruses [44]. These enveloped positive-strand RNA viruses of vertebrates are obviously not related to the orthomyxoviruses, which have a segmented negative-strand RNA genome [20]. They are, however, evolutionary related to each other [45] -albeit distantly [46]- and are currently included as separate subfamilies in the family *Coronaviridae*, Order *Nidovirales* [47]. Coronaviruses can be subdivided further into three genera (five groups) on the basis of genetic and serological criteria [48].

Toroviruses mostly cause mild enteric infections in cattle and swine. In contrast, coronaviruses have been recognized as pathogens of veterinary importance. Human coronaviruses initially seemed to be associated only with mild respiratory infections ("common colds") and hence were considered of modest clinical relevance. This picture has changed radically with the sudden emergence in 2003 of severe acute respiratory syndrome (SARS), which was found to be caused by a novel human coronavirus, SARS-HCoV [49,50], and by the more recent discovery of yet two other new human coronavirus species, HCoV-NL63 [51] and HCoV-HKU1 [52], both of which were isolated from patients with severe pulmonary disease.

Toroviruses and coronaviruses boast RNA genomes of unprecedented size, 28–32 kb, the organization of which is illustrated in Figure 3. The 5′-most two-thirds of these genomes are comprised of two large overlapping open reading frames, which encode the polyproteins from which the various subunits of the viral replicase c.q. transcriptase are derived. Downstream of the replicase genes are the genes for the structural proteins (for a review, see [53]). Note that although the nucleocapsid-(N), membrane- (M) and spike proteins (S) of toro- and coronaviruses go by the same names and acronyms, they do not share primary sequence identity. However, their M proteins, triple-spanning membrane proteins, do share common characteristics [54], as do their S proteins. The latter are class 1 fusion proteins [55–58] and mediate receptor-binding and entry [59,60].

Characteristically, coronaviruses also carry a variable number of genes for group-specific accessory proteins [53]. Among the latter is the HE protein, which is only found in group 2a coronaviruses. Conversely, all toroviruses currently known carry an HE gene.

The HEs of toro- and coronaviruses are class I envelope membrane proteins [30,61–63] of about 400 residues in length and containing 7 to 12 *N*-linked glycosylation sites. HE multimers (see also below) are taken up into virions and, in electron micrographs, appear as small surface projections of 5 to 7 nm, clearly distinct from the prominent 20 nm peplomers, comprised of S (Figure 4) [30,64,65].

The first coronavirus HE gene was discovered by Luytjes *et al.* [43] while sequencing the genome of the mouse hepatitis virus (MHV) strain A59. The predicted gene product displayed 30% sequence identity to the influenza C virus HEF_1. In MHV-A59, however, the HE gene is not expressed: its dedicated mRNA, mRNA2b, is not produced because of a mutation in the "transcription-regulating sequence". Moreover, the gene is interrupted by a nonsense

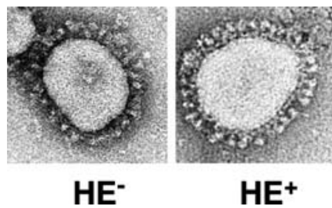


Fig. 4 Virion morphology of isogenic MHV-A59 recombinants, which either express (+) or lack (−) the HE protein (electron micrograph image, courtesy of Jean Lepault). Reprinted from Lissenberg *et al.* [114] with permission

mutation at codon 15 [43,66]. Luytjes and coworkers were quick to grasp, however, that the HE gene might be present and functional in bovine coronavirus (BCoV) and in human coronavirus OC43 (HCoV-OC43), viruses, closely related to MHV, for which in fact a unique 65 kDa envelope glycoprotein, "E3", with hemagglutinin activity had already been described [67–70]. In a collaborative effort with Reinhard Vlasak and Peter Palese, it was demonstrated that BCoV and HCoV-OC43, like influenza C virus, bind to Neu5,9Ac$_2$ [71] and carry virion-associated RDEs with sialate-9-*O*-acetylesterase activity associated with the gp62/E3 surface protein [72] (henceforth named hemagglutinin-esterase [63]). These observations were extended by Schultze and Herrler [73] by showing that BCoV utilizes Neu5,9Ac$_2$ as a receptor determinant for the infection of cultured cells. Formal proof that coronavirus HEs are indeed *O*-acetylesterases was obtained through heterologous expression in mammalian and in insect cells [74–76].

Subsequent studies revealed that not only in MHV-A59, but in many other laboratory strains of MHV, the HE gene is inactivated [77], apparently an *in vitro* artifact resulting from adaptation to propagation in cultured cells. However, some strains, like MHV-S, -JHM and -DVIM, have retained HE expression [66,77] as had actually already been noted in the early 1980's [78–81].

If the presence of an HE gene in coronaviruses already came as a surprise, the discovery of an HE (pseudo)gene in the genome of the equine torovirus (EToV) strain Berne (Berne virus, BEV) was nothing less than mind-boggling [44]. Like MHV-A59, BEV carries an inactive HE gene: about half of its sequence has been deleted. However, the bovine torovirus (BToV) field variant, Breda 2 virus (BRV), possesses an intact HE gene [30] as do all other torovirus field variants known to date [82–84]. The HE proteins of toro-, corona- and influenza C viruses are evolutionary equidistant with ∼30% sequence identity in all directions [30,43,44]. Initial studies demonstrated that BRV HE, like HEF [85], hydrolyzes the synthetic substrate α-naphtyl acetate and hence displays esterase activity [30]. By testing their reactivity towards free and glycosidically-bound Sias, we have now provided formal evidence that also the torovirus HEs are sialate-*O*-acetylesterases [86].

The occurrence of closely related HE sequences in three different groups of viruses must be ascribed to heterologous RNA recombination [43,44], but the source of the HE gene is unknown. In principle, the gene might have originated in any of the three viruses and subsequently have spread to the others via horizontal gene transfer. It is quite possible, however, that the HE module arose in an as yet unidentified donor, cellular or viral, to be incorporated into the genomes of influenza C virus, toroviruses and the group 2 coronaviruses through independent recombination events.

In phylogenetic trees, the HE proteins of corona-, toro-, and influenza C viruses divide into three separate monophyletic clusters (Figure 5), a topology consistent with the notion that in each virus the introduction of the HE module occurred only once [86]. In the case of the group 2 coronaviruses, the HE module seems to have been obtained after the early split-off of SARS-HCoV, which lacks an HE gene [49,50,86,87], but before the acquisition of accessory genes 2A and 5; the latter are absent from the genome of HCoV-HKU1 (Figure 3) [52].

Recently, a novel hemagglutinin-esterase protein was discovered in infectious salmon anemia virus (ISAV), an *Orthomyxovirus* of teleosts [88,89]. Sequence similarity between the ISAV HE and those of the influenza *C* and nidoviruses is limited to the regions containing the active site residues [88]. Nonetheless, this important new finding reveals that the viral HEs belong to a protein family more diverse and widespread than appreciated so far.

Sequence diversity among nidovirus HE proteins

Amongst corona- and toroviruses, sequence divergence in the HE proteins is considerable, up to 50 and 30%, respectively. The coronavirus HEs branch into five lineages (A through E), of which, saliently, those of groups A and B both originate from MHV strains. The HE proteins of toroviruses likewise divide into two porcine and two bovine lineages (Figure 5); the HE protein reported for human torovirus [90,91] is a peculiar outlier, more related to that of BToV-BRV (74% sequence identity) than to any of the other torovirus HEs characterized thus far (not shown).

Nidoviruses display high frequency homologous RNA recombination both *in vitro* and in the field. Hence, the phylogenetic relationships in Figure 5 reflect the evolutionary history of the *HE modules* and explicitly not that of the *viruses* from which these genes were derived. Indeed, ancestors of extant bovine and porcine field strains apparently replaced their original HE sequences by new ones via intertypic RNA recombination with related but hitherto unidentified toroviruses, thus giving rise to BToV B150- and (presumably) PToV *P*-MAR-type variants [83]. Similar recombination events took place during the divergence of the

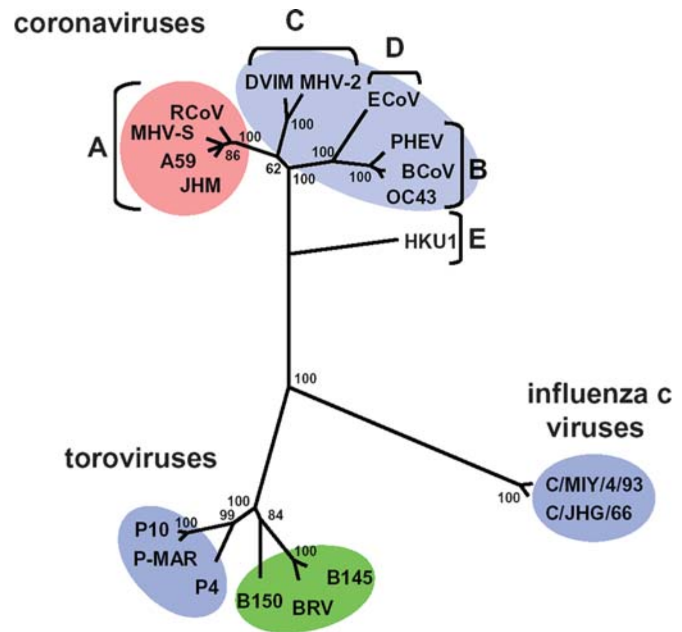


Fig. 5 Neighbour-Joining tree illustrating the phylogenetic relationships among influenza C- and nidovirus HE proteins. The five coronavirus HE lineages are indicated A through E. Colored clouds indicate HEs with sialate-9-*O* (blue), -4-*O*- (pink) or -7(8)9-*O*-acetylesterase activity (green). Confidence values calculated by bootstrapping (1000 replicates) are indicated at the major branching points. Adapted from Smits *et al*. [86]

group 2a rodent coronaviruses, resulting in MHV strains with either an A-type or B-type HE gene [86].

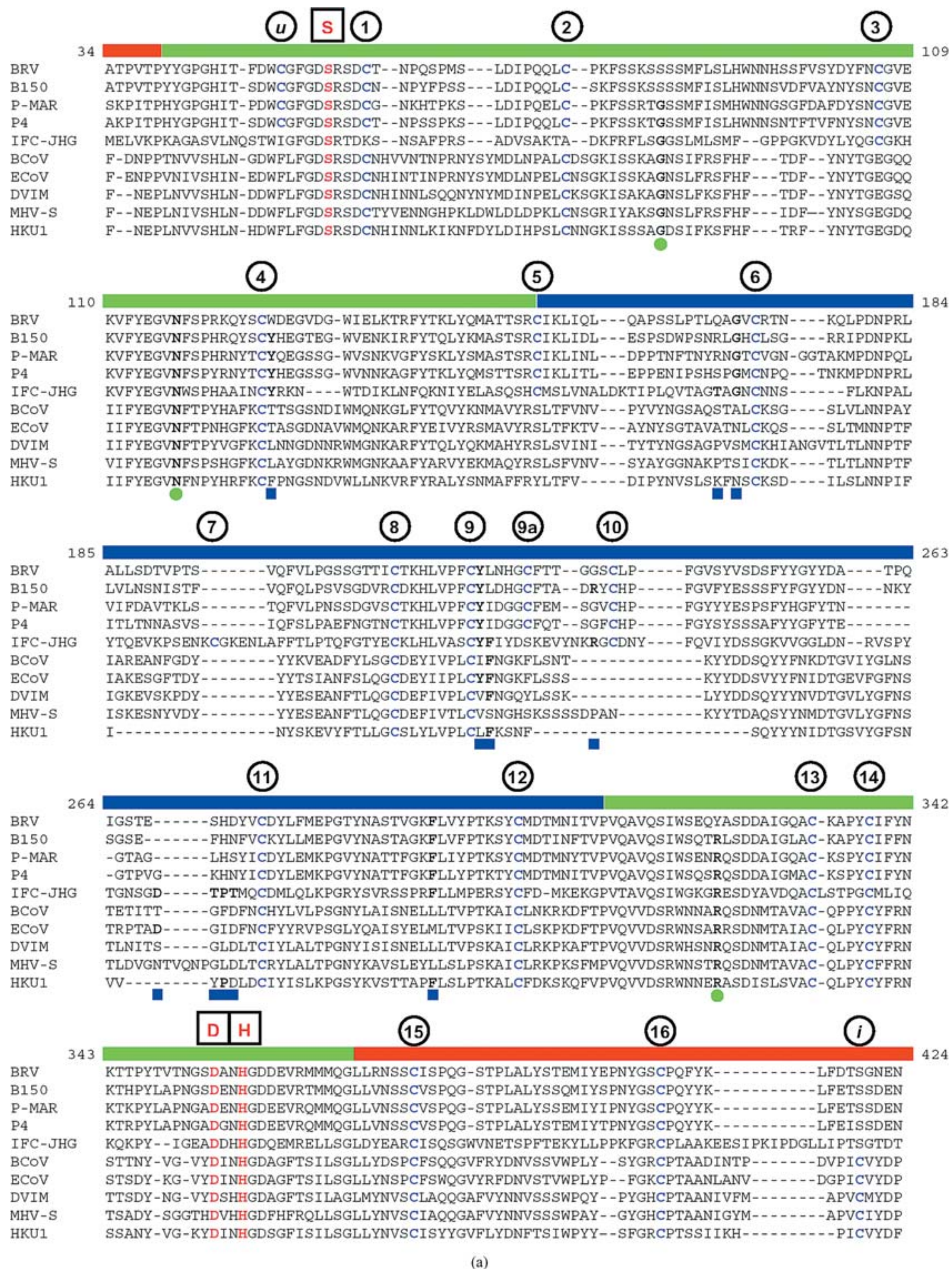
Structural characteristics of nidovirus HE proteins

Nidoviral HE proteins are membrane-anchored via a C-terminal hydrophobic domain, which in the primary sequence of HEF would correspond to the fusion peptide at the N-terminus of HEF_2. Segments, corresponding to HEF_1 domains E and R and to what might be vestigial remnants of domain F [31], can be readily distinguished (Figure 6a) and there is little doubt that the overall 3D structure of nidoviral HEs will be highly similar to that of HEF_1. This is emphasized by the tight conservation of disulfide bonds (Figure 6b). Most disulfides in HEF_1 are also present in the corona- and torovirus HEs. There are some noteworthy differences, however. The single cysteine residue Cys^{6} at the N-terminus of HEF_1, which forms an intermolecular disulfide bond with Cys^{137} of HEF_2 [31], is absent in nidovirus HE proteins. In coronavirus HEs, bonds equivalent to those between HEF Cys^{106} and Cys^{151} (C3–C5; E domain) and between Cys^{196} and Cys^{238} (C7–C10; R domain) are lacking, whereas in torovirus HEs, the C7–C10 bond apparently has been replaced by a disulfide bond between C10 and a new cysteine residue, C9a. In turn, a surface loop, which in HEF_1 walls the esterase substrate binding pocket (residues 61–77), is closed by a disulfide bond (C1–C2) in the nidoviral HEs, but not in HEF_1 (Figure 6) [31].

In the E domain, the catalytic Ser-His-Asp triad is strictly conserved. Also conserved are residues equivalent to HEF Gly^{85}, Asn^{117} and Arg^{322}, though in the BToV HEs, Gly^{85} is replaced by Ser. Most variation between and among orthomyxo- and nidoviral HEs is seen in the receptor domain *R* with numerous amino acid substititions, large insertions and/or deletions and even rearrangement c.q. loss of disulfide bonds (Figure 6). There is no obvious conservation of the HEF_1 receptor-binding site in the nidovirus proteins. This raises the question whether there is a separate receptor-binding site in nidovirus HEs and if so, what it might look like.

Another interesting aspect of the HE(F) proteins concerns the differences in their quaternary structures. Whereas the influenza C virus HEF is trimeric, coronavirus HE proteins form disulfide-bonded homodimers [68,69,79,81,92]. According to the coronavirus HE disulfide map (Figure 6b), a single cysteine located immediately upstream of the transmembrane anchor would remain without a partner in the monomer. This residue, which is absent in the HEs of influenza C virus and toroviruses, is the obvious candidate for intermolecular disulfide bond formation. Whether the HE spikes on the surface of coronavirions consists of HE dimers or multimers thereof remains to be determined.

Undoubtedly, torovirus HE proteins also assemble into multimeric complexes, though apparently through non-covalent interactions. No disulfide-linked oligomers were detected in cells heterologously expressing BToV-BRV HE or in radioiodinated sucrose-gradient purified BToV-BRV preparations [30]. One Cys residue, Cys^{18}, which is conserved in all torovirus HE proteins but absent in those of corona- and influenza C virus, is predicted to be without a partner in the monomer (Figure 6b). However, as this residue would be lo-



(a)

Fig. 6 (a) Structure-based alignment of the ectodomains of toro- and coronavirus HE proteins to influenza C virus HEF_1. Sequence segments are color-coded by domains (F, red; E, green; R, blue). Boxes marked S, D and H indicate the catalytic active site residues Ser, His and Asp. Also indicated by green dots are the positions of HEF_1 residues Gly^{85} and Asn^{117} of which the NH group and the side chain, respectively, contribute to the oxyanion hole, and of Arg^{322}, which assists in substrate binding. HEF_1 residues involved in receptor binding (see Figure 2 and text) are indicated by blue squares. Cysteine residues are indicated by numbers in circles, with the numbering corresponding to that in Figure 6b. "*u*" indicates a cysteine residue unique to torovirus HEs (Cys^{18}), which presumably remains unpaired; "*i*" indicates a cysteine residue, unique to coronavirus HEs, which is predicted to remain without a partner in the monomer and hence is the most likely candidate for intramolecular disudlfide formation. (b) Schematic representation of the disulfide-bonded structure of influenza C virus HEF and the predicted structures of the corona- and torovirus HEs. Cysteine residues are numbered as in Figure 6a. Sequence segments are colored by domains (F, red; E, green; R, blue). Transmembrane domains in the nidovirus HEs are indicated by grey boxes. The arrowhead indicates the HEF cleavage site

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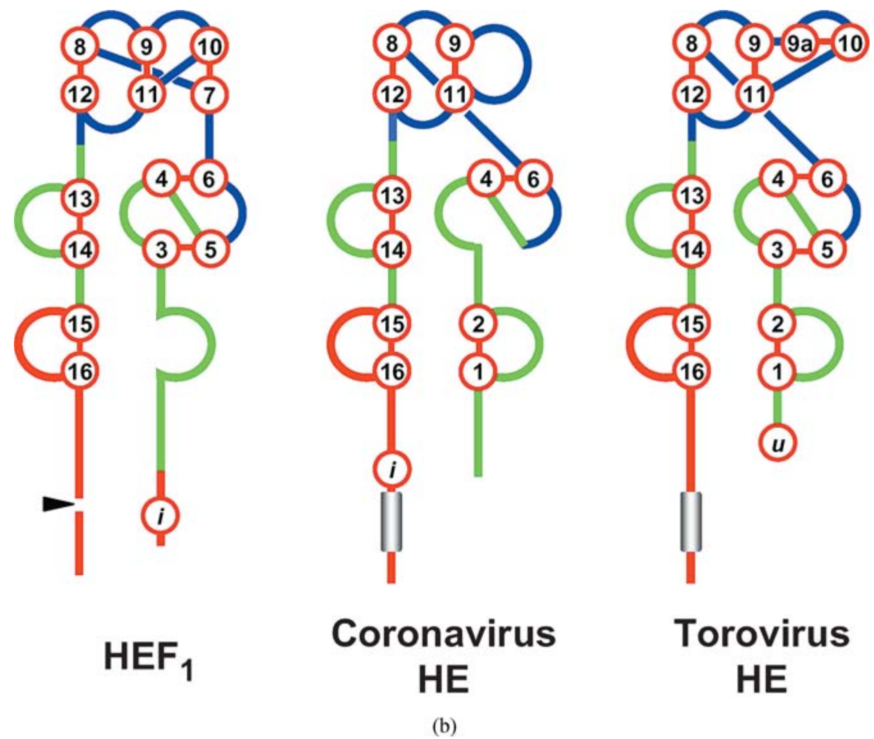
Fig. 6 (*Continued*)



(b)

cated within the interior of the protein, in close proximity of the catalytic pocket, it presumably remains unpaired.

Differences in substrate specificity between nidoviral HE proteins

The first coronaviral HE proteins to be analyzed in terms of substrate specificity, *i.e.* those of BCoV, HCoV-OC43 and porcine hemagglutinating encephalomyelitis virus (PHEV) were all sialate-9-*O*-acetylesterases thus resembling HEF [71,72,93]. Indications for the existence of HEs of different specificity came from studies on puffinosis virus (PuCoV), allegedly a virus isolate from certain seabirds (Manx shearwater, *Puffinus puffinus*), but almost certainly an MHV strain of laboratory mice as based upon its isolation history, entailing passage in mouse brain and cultured murine cells [94], and its (phylo)genetic signature [86,95,96]. The PuCoV HE protein displayed esterase activity when assayed with synthetic *O*-acetylated substrates, but no detectable activity towards natural substrates containing Neu5,9Ac$_2$ [95]. Subsequent studies showed that the HE of PuCoV, those of two other MHV strains (S and JHM) and also that of the related rat coronavirus (RCoV) are in fact sialate-4-*O*-acetylesterases, which efficiently convert free as well as glycosidically-bound Neu4,5Ac$_2$ to Neu5Ac (Figure 1) [96–98].

Interestingly, however, not all rodent coronaviruses express an HE of this specificity: murine coronaviruses actually occur in two varieties with some strains preferring 4-*O*- and others 9-*O*-acetylated Sias. MHV-DVIM, the only hemagglutinating MHV strain identified so far [65,80,99], binds to Neu5,9Ac$_2$-receptor determinants on rat erythrocytes (A.L.W. van Vliet, M.A. Langereis and R.J. de Groot, *unpublished observations*) and correspondingly possesses a "B-type" HE with sialate-9-*O*-acetylesterase activity [86]. MHV strains with sialate-4-*O*-acetylesterase activity (A-type HEs) do not cause hemagglutination of any type of red blood cell tested [99], but they do bind specifically to Neu4,5Ac$_2$-containing glycoconjugates in solid phase assays [96,97].

Among the toroviruses, the porcine field isolates produce HE proteins with sialate-9-*O*-acetylesterase activity. Bovine toroviruses, however, express HE proteins with a preference for the di-*O*-acetylated Sia Neu5,7,9Ac$_3$, suggesting that these viruses have adapted to using this Sia subtype as (co)-receptor [86]. The choice for a particular type of Sia may be determined by the ubiquity of this receptor determinant in the natural host and by its tissue and/or cell distribution. It is tempting to speculate that at least some of the changes in viral preference for distinct Sia subsets occurred as nidoviruses adapted to new hosts and/or new target tissues.

It is of note that so far nidoviral HEs have been tested for specificity using a limited set of Sia substrates only. Hence, it cannot be excluded that even among those enzymes, which are currently considered to be of identical specificity, subtle differences exist in preference for particular Sia subsets. For instance, differences in substrate preference towards more exotic, multiply-modified Sias (for example Sias in which *O*-acetylation is combined with other substituents at C4, C7 and C8) may have gone undetected so far. Strains of

HCoV-OC43 and BCoV reportedly differ in their receptor preference for α2-6-linked 9-*O*-acetylated sialic acids [100,101], traits, which may well be mirrored by the substrate preferences of their sialate-*O*-acetyleseterases.

The issue of substrate fine-specicity may be especially relevant in the case of the HE of MHV-DVIM, which was identified as a sialate-9-*O*-acetylesterase in assays with free Sia's as substrates. In further support of its proposed specificity, the DVIM RDE readily destroyed BCoV receptors on rat and chicken erytrocytes as well as those on MDBK-I cells [86] (M.A. Langereis, A.L. van Vliet and R.J. de Groot, *unpublished observations*). Puzzlingly, however, the glycosidically-bound Neu5,9Ac_2 moieties of bovine submaxillary mucin are not efficiently de-*O*-acetylated [86,102]. Ideally, future studies should entail systematic quantitative analysis of HE reactivity towards libraries of well-defined Sia-containing glycoconjugates.

Nidovirus HE proteins: RDEs, Sia-specific viral lectins or both?

According to their designation and the prevailing view in the field, the nidovirus HEs should serve not only as RDEs, but also as hemagglutinins or at least as Sia-specific lectins. There is ample evidence in support: (i) mammalian cells, heterologously expressing HEs of BCoV, MHV-JHM, or BToV HE, display hemadsorption [74,76]; L.A.H.M. Cornelissen and R.J. de Groot, *unpublished observations*), (ii) the HE proteins of BCoV, PHEV and MHV-DVIM, purified from virions by detergent extraction and sucrose-gradient centrifugation, agglutinate rat and mouse erythrocytes in a Neu5,9Ac_2-dependent fashion [92,93,102,103], (iii) the hemagglutinating activity of BCoV and MHV-DVIM can be inhibited by HE-specific monoclonal antibodies [67,104–106], (iv) bromelain-treatment of BCoV and HCoV-OC43 virions results in degradation of the S protein, but leaves both the HE and the hemagglutinating activity unaffected [69,70].

Herrler and colleagues [100,103] have argued, however, that the coronaviral HEs might play only a modest role in virus attachment to Sia's. The spike proteins of BCoV and HCoV-OC43 are potent Neu5,9Ac_2-specific lectins and, when purified by octyl-glucoside-extraction and sucrose gradient centrifugation, agglutinate not only murine and rat erythrocytes, which are rich in 9-*O*-acetylated Sia's, but also chicken erythrocytes, on which these receptors are relatively sparse [103]. The HE proteins, purified according to the same procedure, also bind to Neu5,9Ac_2, but agglutinate only the red blood cells of rodents [93,100,103]. These findings were interpreted to indicate that, in comparison to S, HE is only a minor hemagglutinin, and that S is responsible for primary attachment of virus to the cell surface. The envisaged functional dichotomy, with one surface protein mediating receptor-binding and entry, and the other primarily acting as RDE, would be reminiscent of the situation in influenza A and B viruses. This model is at odds, however, with findings of Sugiyama *et al.* [102], who, by comparing the biological properties of the purified surface proteins of MHV-DVIM, assigned the hemagglutinating activity to HE and not to S. The experimental approaches taken by the groups of Herrler and Sugiyama, which involved the analysis of purified virion proteins, have as a caveat that receptor-binding activity might have been lost or compromised during the purification process. In an attempt to clarify the issue, we have employed reverse genetics to separately introduce the S and HE genes of MHV-DVIM into the genome of MHV-A59 (i.e. a non-hemagglutinating strain). Only the recombinant viruses expressing DVIM HE agglutinated rat erythrocytes, indicating that indeed, in MHV-DVIM, the HE and not the S protein is the hemagglutinin (M.A. Langereis, A.L. van Vliet and R.J. de Groot, *manuscript in preparation*).

Surveying the combined data, it appears that there are important differences between group 2a coronaviruses with respect to Sia receptor usage. Murine coronaviruses, irrespective of whether they encode an HE protein with sialate-4- or sialate-9-*O*-acetylesterase activity, all employ isoforms of the murine carcinoembryonic antigen cell adhesion molecule 1a (mCEACAM) as their principal receptors [107–112]. Entry of cultured cells relies exclusively on binding of S to mCEACAM. HE expression is dispensable for MHV propagation *in vitro* [43,77] and *in vivo* [113] and, in many cultured cells, even provides a selective disadvantage [114]. Remarkably, however, Taguchi *et al.* [115] observed that HE-expressing variants of MHV-JHM were selected for both during propagation in cultured neural rat cells as well as in the brains of infected rats. In nature, HE genes are maintained: rather then lost they are exchanged among MHV variants through homologous RNA recombination [86]. Thus, under field conditions, HE expression must be beneficial to the virus. Apparently, *in vivo*, MHVs use Sia as co-receptor or initial attachment factor with virion binding to Sia receptor determinants most likely being mediated by HE. In support of this notion, we recently found that HE -irrespective of whether it retains enzymatic activity or not- promotes dissemination of MHV in the brains of intra cranially-inoculated mice [116].

BCoV and HCoV-OC43 seem to have taken their adaptation to Sia receptor determinants even one step further and use 9-*O*-acetylated Sias as their primary receptors [73,117]; M.A. Langereis, A.L. van Vliet, and R.J. de Groot, *unpublished observations*). Although possibly a second receptor is involved [117], the binding of S to Neu5,9Ac_2 receptor determinants appears to be required and essential for successful entry in cultured cells: (i) MHV, when pseudotyped with the S but not the HE protein of BCoV, displays an altered host cell tropism and can now infect BCoV-permissive MDBK I cells, which are normally MHV-resistant [118]. (ii)

BCoV-infection *in vitro* can be prevented by prior treatment of cultured cells with neuraminidase or with viral sialate-*O*-acetylesterases [73] (M.A. Langereis, A.L. van Vliet, and R.J. de Groot, *unpublished observations*). Yet, also in the case of BCoV and HCoV-OC43, it seems premature to draw definitive conclusions on the roles of HE and S in Sia-dependent attachment to host cells. The S protein might be the more potent lectin, the number of HE and S spikes on the virion surface should also be taken into account. If, as it would appear, the HE spikes outnumber the S spikes, they might through multiple low affinity, high avidity interactions with Neu5,9Ac$_2$ receptor determinants significantly contribute to virion attachment after all.

Where from here?

Evidently, the function of the HE proteins in nidovirus infections merits further investigation. In particular, their contribution to host cell tropism, viral spread and pathogenesis should be explored. With protocols for reverse genetics available, MHV provides the most attractive model system. In this context, it will be particularly interesting to assess *in vivo* differences relating to Sia receptor-preference. Another important though challenging research venue is the crystallization of nidovirus HEs. Insight into the 3D-structure of these fascinating proteins may further our understanding of HE evolution and answer intriguing questions concerning the presence of Sia receptor binding sites, the molecular basis for substrate- and receptor specificity, and the stoichiometry of the HE spikes.

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