

Coronavirus receptor switch explained from the stereochemistry of protein–carbohydrate interactions and a single mutation

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Hemagglutinin-esterases (HEs) are bimodular envelope proteins of orthomyxoviruses, toroviruses, and coronaviruses with a carbohydrate-binding “lectin” domain appended to a receptor-destroying sialate-*O*-acetyl esterase (“esterase”). In concert, these domains facilitate dynamic virion attachment to cell-surface sialoglycans. Most HEs (type I) target 9-*O*-acetylated sialic acids (9-*O*-Ac-Sias), but one group of coronaviruses switched to using 4-*O*-Ac-Sias instead (type II). This specificity shift required quasisynchronous adaptations in the Sia-binding sites of both lectin and esterase domains. Previously, a partially disordered crystal structure of a type II HE revealed how the shift in lectin ligand specificity was achieved. How the switch in esterase substrate specificity was realized remained unresolved, however. Here, we present a complete structure of a type II HE with a receptor analog in the catalytic site and identify the mutations underlying the 9-*O*- to 4-*O*-Ac-Sia substrate switch. We show that (i) common principles pertaining to the stereochemistry of protein–carbohydrate interactions were at the core of the transition in lectin ligand and esterase substrate specificity; (ii) in consequence, the switch in *O*-Ac-Sia specificity could be readily accomplished via convergent intramolecular coevolution with only modest architectural changes in lectin and esterase domains; and (iii) a single, inconspicuous Ala-to-Ser substitution in the catalytic site was key to the emergence of the type II HEs. Our findings provide fundamental insights into how proteins “see” sugars and how this affects protein and virus evolution.

coronavirus | hemagglutinin-esterase | sialic acid | crystal structure | sialate-*O*-acetyl esterase

Among host cell surface determinants for pathogen adherence, sialic acids (Sias) rank prominently (1, 2). Representatives of at least 11 families of vertebrate viruses use Sia as primary entry receptor and/or attachment factor (3, 4). Viral adherence to sialoglycans, however, comes with inherent complexities related to (i) the sheer ubiquity of receptor determinants that may act as “decoys” when present on off-target cells and non-cell-associated glycoconjugates, and (ii) the dense clustering that is characteristic to glycotopes and that may augment the apparent affinity of ligand–lectin interactions by orders of magnitude (5, 6). Viruses may avoid inadvertent virion binding to nonproductive sites by being selective for particular sialoglycan subtypes so that attachment is dependent on Sia linkage type, the underlying glycan chain, and/or the absence or presence of specific postsynthetic Sia modifications (2, 7, 8). Moreover, as an apparent strategy to evade irremediable binding to decoy receptors, viral sialolectins typically are of low affinity, with dissociation constants in the millimolar range (reviewed in ref. 3). In consequence, virion–Sia interactions are intrinsically dynamic and the affinity of the virolectins would appear to be fine-tuned such as to ensure reversibility of virion attachment. In most viruses, reversibility is exclusively subject to the lectin–ligand binding equilibrium. Some, however, take this principle one step further by

encoding virion-associated enzymes to promote catalytic virion elution through progressive local receptor depletion (3, 4).

In lineage A betacoronaviruses (A-βCoVs), a group of enveloped positive-strand RNA viruses of human clinical and veterinary relevance (9), catalysis-driven reversible binding to *O*-acetylated Sias (*O*-Ac-Sias) is mediated by the hemagglutinin-esterase (HE), a homodimeric type I envelope glycoprotein (10–15). HE monomers resemble cellular carbohydrate-modifying proteins (16, 17), in that they have a bimodular structure with a lectin appended to the enzyme domain. The lectin domain mediates virion attachment to specific *O*-Ac-Sia subtypes with binding hinging on the all-important sialate-*O*-acetyl moiety, whereas removal of this *O*-acetyl by the catalytic sialate-*O*-acetyl esterase (“esterase”) domain results in receptor destruction (18–21).

Intriguingly, HE homologs also occur in toroviruses (22–25) as well as in three genera of orthomyxoviruses (*Influenza virus C*, *Influenza virus D*, and *Isavirus*) (26–32), but, among coronaviruses, exclusively in A-βCoVs (9). HE was added to the proteome of an A-βCoV common progenitor through horizontal gene

Significance

A wide variety of vertebrate viruses, representative of at least 11 families, use sialic acid (Sia) for host cell attachment. In betacoronaviruses, the hemagglutinin-esterase envelope protein (HE) mediates dynamic attachment to *O*-acetylated Sias. HE function relies on the concerted action of carbohydrate-binding lectin and receptor-destroying esterase domains. Although most betacoronaviruses target 9-*O*-acetylated Sias, some switched to using 4-*O*-acetylated Sias instead. The crystal structure of a “type II” HE now reveals how this was achieved. Common principles pertaining to the stereochemistry of protein–carbohydrate interactions facilitated the ligand/substrate switch such that only modest architectural changes were required in lectin and esterase domains. Our findings provide fundamental insights into how proteins “see” sugars and how this affects protein and virus evolution.

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Data deposition: The three crystal structures have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 4ZXN (RCOV-NJ HE⁰), 5JIF (MHV-DVIM HE), and 5JIL (RCOV-NJ HE⁰ in complex with 4-*N*-acetylated sialic acid)].

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transfer and apparently originated from a 9-*O*-Ac-Sia-specific hemagglutinin-esterase fusion protein (HEF) resembling those of influenza viruses C and D (10, 19). The acquisition of HE, either or not in conjunction with that of other accessory proteins like ns2a (33), may well have sparked the radiation of the A- β CoV. At any rate, their expansion through cross-species transmission was accompanied by evolution of HE, apparently reflecting viral adaptation to the sialoglycomes of the novel hosts (14). For example, the HE of bovine coronavirus (BCoV) preferentially targets 7,9-di-*O*-Ac-Sias, a trait shared with the HEs of bovine toroviruses (8, 24). The most dramatic switch in *O*-Ac-Sia specificity occurred in the murine coronaviruses (MuCoVs), a species of A- β CoVs in mice and rats (9). Two MuCoV biotypes can be distinguished on the basis of their HE (14) with one group of viruses using the prototypical attachment factor, 9-*O*-Ac-Sia (type I specificity) (24), and the other exclusively binding to Sias that are *O*-acetylated at carbon atom C4 (4-*O*-Ac-Sia) (type II specificity) (15, 24, 34, 35). Although deceptively similar in nomenclature and acronyms, 9-*O*- and 4-*O*-Ac-Sias are quite different in structure (Fig. 1A), particularly when taking into account that the sialate-*O*-acetyl is paramount to protein recognition. Thus, in molecular terms, the shift in ligand/substrate preference would seem momentous. As rules of virus evolution would predict, and in accordance with the phylogenetic record (14), the transitions in ligand and substrate specificity that required coevolution of two distinct protein domains (i.e., lectin and esterase) must have occurred swiftly and, although not necessarily simultaneously, at least within a narrow time frame.

Previous analysis of an HE structure of MuCoV type II strain S revealed how the shift in Sia specificity was accomplished for the lectin domain (21). Its comparison with the (type I) HE of BCoV [a member of species *Betacoronavirus-1* distantly related to MuCoV (9)] allowed for a rough reconstruction of the remodeling of the lectin's carbohydrate binding site (CBS). The catalytic site, however, was disordered (21), and hence the question of how the switch in substrate specificity was brought about remains unresolved. We now present fully resolved crystal structures of a type II HE, free or with ligand/substrate analogs in the Sia binding sites of both lectin and esterase domain. To allow for a minute side-by-side comparison, we also determined the structure of the esterase domain of a closely related type I MuCoV HE. Comparative structural analysis corroborated by structure-guided mutagenesis revealed the crucial changes that underlie the substrate specificity switch and thus established the structural basis for type II substrate selection. Our findings indicate that basic principles pertaining to the stereochemistry of protein-carbohydrate interactions were at the core of the transition in lectin ligand and esterase substrate specificity. We propose that, within this context, a single inconspicuous amino acid substitution in the catalytic site—in essence, the mere introduction of an oxygen atom—was key to the emergence of the type II HEs.

Results and Discussion

Structure Determination and Overall Structures. The HE ectodomains of murine coronavirus strains MHV-DVIM (type I) and RCoV-NJ (type II), either intact or rendered catalytically inactive through active-site Ser-to-Ala substitutions (HE⁰), were expressed as thrombin-cleavable Fc fusion proteins. The expression products retained full biological activity as was demonstrated by solid-phase lectin-binding assays and receptor destruction assays with bovine submaxillary mucin (BSM) and horse serum glycoproteins (HSGs) (Fig. 1B); these sialoglycoconjugates carry 9-*O*-Ac- and 4-*O*-Ac-Sias, respectively (36, 37), and were used to assess esterase specificity throughout.

Crystals of MHV-DVIM HE, and of RCoV-NJ HE⁰, free or in complex with the nonhydrolysable ligand/substrate analog 4,5-di-*N*-acetylneuraminic acid α -methylglycoside (α -4-*N*-Ac-Sia), diffracted to 2.0, 2.2, and 1.85 Å, respectively. Structures were solved by molecular replacement using MHV-S HE [Protein Data

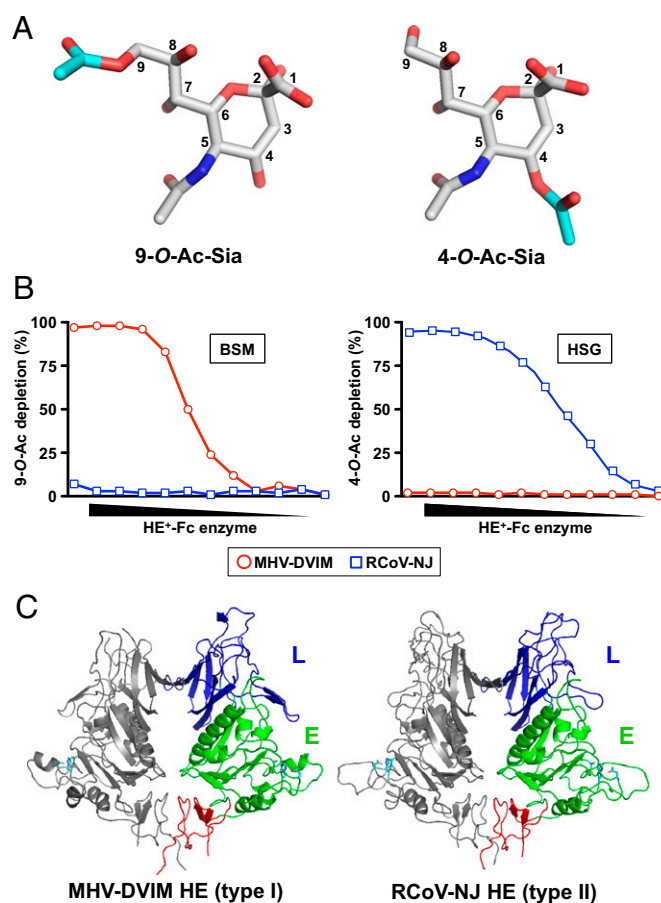


Fig. 1. (A) Stick representation of 9-*O*-Ac-Sia and 4-*O*-Ac-Sia. *O*-Ac moieties are depicted with carbon atoms in cyan. (B) Substrate specificity of MHV-DVIM HE (red circles) and RCoV-NJ HE (blue squares). BSM (Left) and HSG (Right) were coated on MaxiSorb plates and incubated with twofold serial dilutions (starting at 100 ng/μL) of enzymatically active HE-Fc fusion proteins. Loss of 4-*O*- and 9-*O*-Ac-Sias (indicated by percentual depletion on the y axis) was assessed by solid-phase lectin-binding assay with enzymatically inactive virolectins MHV-S HE⁰-Fc and PToV-P4 HE⁰-Fc, respectively, with virolectin concentrations fixed at 50% maximal binding. (C) Cartoon representation of the crystal structures of the RCoV-NJ HE and MHV-DVIM HE dimers. The Left monomer is colored gray, the other by domain: lectin domain (L, blue); esterase domain (E, green) with Ser-His-Asp active site triad (cyan sticks); membrane proximal domain (red).

Bank (PDB) ID code 4C7W; for RCoV-NJ HE] and BCoV-Mebus HE (PDB ID code 3CL5; for MHV-DVIM HE) as search models. For crystallographic details, see Table 1.

Overall, the murine coronavirus HEs closely resemble those of other nidoviruses, assembling into homodimers and with monomers displaying the characteristic domain organization (Fig. 1C) (19, 20). For RCoV-NJ HE, complete structures were determined. In the case of MHV-DVIM HE, the lectin domain was partially disordered, but the structure of the esterase domain was resolved.

The lectin domain of RCoV-NJ HE is virtually identical to that of (type II) MHV-S HE (21) (rmsd on main chain C α atoms: 0.31 Å; Table S1; for a sequence alignment of representative type I and II HEs, see Fig. S1). The same holds for the binding mechanism and topology of the ligand (Figs. S24 and S3B). One notable difference is in the lectin domain's metal-binding site, a signature element of coronavirus HEs (19). That of RCoV-NJ contains Na⁺ rather than K⁺ as inferred from the bond lengths to the coordinating amino acids (Asp²²⁵, Ser²²⁶, Gln²²⁷, Ser²⁷³, Glu²⁷⁵, and Leu²⁷⁷), B factors, and abundance in crystallization solution (Tables

Table 1. Data collection and refinement statistics

Data collection and refinement	MHV-DVIM HE	RCoV-NJ HE free	RCoV-NJ HE complex
Data collection			
Synchrotron	ESRF	SLS	ESRF
Beamline	ID23-1	PX	ID23-2
Wavelength, Å	0.9999	0.9999	0.8729
Space group	P2 ₁ 2 ₁ 2 ₁	C222 ₁	C222 ₁
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> , Å	88.52, 88.82, 122.16	60.71, 184.37, 76.90	57.09, 184.59, 78.08
α , β , γ , °	90, 90, 90	90, 90, 90	90, 90, 90
Resolution range, Å*	44.41–2.00 (2.03–2.00)	61.2–2.2 (2.27–2.20)	54.54–1.85 (1.89–1.85)
Total no. reflections	601,769 (20257)	92,318 (8952)	107,080 (5853)
No. unique reflections	65,139 (2878)	22,281 (2049)	33,539 (2066)
<i>R</i> _{merge}	0.096 (1.184)	0.109 (0.68)	0.106 (0.519)
<i>I</i> / σ <i>I</i>	12.5 (2.3)	8.5 (2.2)	6.2 (1.8)
Redundancy	9.24 (7.0)	4.1 (4.4)	3.2 (2.8)
Completeness, %	99.2 (90.9)	99.6 (100)	94.4 (95.7)
CC(1/2)	0.999 (0.747)	0.995 (0.815)	0.990 (0.577)
Refinement			
<i>R</i> _{work} / <i>R</i> _{free}	0.1990/0.2264	0.2319/0.2783	0.1851/0.2006
No. atoms			
Protein	5,708	2,929	3,058
Water/other ligands	223/463	89/86	186/182
Average B/Wilson B, Å ²	52.0/42.5	40.99/25.4	12.5/22.5
Rms deviations			
Bond lengths, Å	0.018	0.0094	0.007
Bond angles, °	1.949	0.9254	1.300
Ramachandran plot			
Favored, %	96.6	95.0	97.0
Allowed, %	3.4	5.0	3.0
Outliers, %	0	0	0

*Numbers between brackets refer to the outer resolution shell.

S2 and S3). Its structure, however, is fully conserved, with all key residues in RCoV-NJ HE aligning with those in MHV-S HE (Fig. S2B). It would thus appear that the metal-binding site in the type II lectin domain can be occupied by either Na⁺ or K⁺ without major consequences for protein structure and function.

The esterase domain of the RCoV-NJ HE is strikingly similar to those of MHV-DVIM and BCoV-Mebus HE (Fig. S1; rmsd of 0.25 Å on main-chain C α atoms for all three combinations, Table S1), despite the difference in substrate specificity. As was predicted from primary sequence similarity [66% identity overall between MHV-DVIM and RCoV-NJ HE and 70% in the esterase domain (14)] and confirmed by present structural data, the shift in substrate-specificity from 9- to 4-*O*-Ac-Sia required minimal architectural changes. A crystal structure of RCoV-NJ HE⁰ complexed with 4,5-di-*N*-Ac-Sia was obtained by soaking at high Sia concentrations (100 mM) and low temperature (4 °C) to allow for the stabilization of low-affinity interactions. The electron density map revealed a well-defined substrate analog molecule (Fig. S3) bound in the active site.

All Elements of the Ancestral Type I Catalytic Center Are Conserved in Sia-4-*O*-Ac-Specific Type II HEs. The nidoviral and orthomyxoviral esterase domains form a separate family in the c.23.10 Ser-Gly-Asn-His (SGNH) superfamily of esterases and acetylhydrolases (18, 38). These enzymes are characterized by an $\alpha\beta$ domain organization with a central five-stranded parallel β -sheet, and by strict topological conservation of catalytic SGNH residues (Fig. 2A). As illustrated in Fig. 2B for MHV-DVIM HE, the Ser and His residues, together with Asp form a catalytic triad, arranged in a linear array. Flanking the catalytic triad is a hydrophobic specificity pocket (P1) to accommodate—in *O*-acylesterases—the

methyl group of the target Sia-*O*-acetylate. The conserved Gly and Asn residues located along the upper rim of this pocket contribute through main-chain and side-chain amides, respectively, to create an oxyanion hole in combination with the main-chain amide of the active site Ser (Fig. 2A, C, and D) (18, 39, 40).

The viral esterase domains differ from other SGNH hydrolases by the presence of a second hydrophobic pocket (P2) on the opposite side of the catalytic triad (18–21). In sialate-9-*O*-acylesterases, this pocket serves to harbor the (hydroxy)methyl group of the Sia-5-*N*-acyl moiety (18). Another hallmark is a strategically positioned Arg (Arg³⁰⁵ in DVIM HE), the side chain of which extends into the catalytic center (Fig. 2A, C, and D). Although not essential for catalysis per se, this Arg is of overriding importance for substrate binding and, in consequence, for the efficient cleavage of glycosidically bound 9-*O*-Ac-Sias (20). Its side chain's head group engages in a bidentate hydrogen bond interaction with the Sia-carboxylate (18, 39), thus fixing the Sia pyranose ring in a proper orientation such that the Sia-9-*O*-acetyl is brought in close proximity of the active-site nucleophile. As we observed for torovirus type I HEs (20), substitution in DVIM HE of Arg³⁰⁵ by Ala abrogates enzymatic activity toward natural substrates (Fig. 2E), but does not affect cleavage of the synthetic substrate *p*-nitrophenyl acetate (*p*NPA) (Fig. 2F).

Remarkably, all elements of the ancestral/archetypical Sia-9-*O*-AE catalytic center, including P1 and P2 pockets, are present in Sia-4-*O*-Ac-specific MuCoV type II HEs, with a near-perfect alignment in MHV-DVIM and RCoV-NJ HEs of all residues known to control sialate-9-*O*-acylesterase activity (Fig. 2A). With the enzymatic mechanism and all main structural elements for catalysis preserved, a shift in esterase specificity from 9- to 4-*O*-acetylated Sias could only have been effectuated by changing the

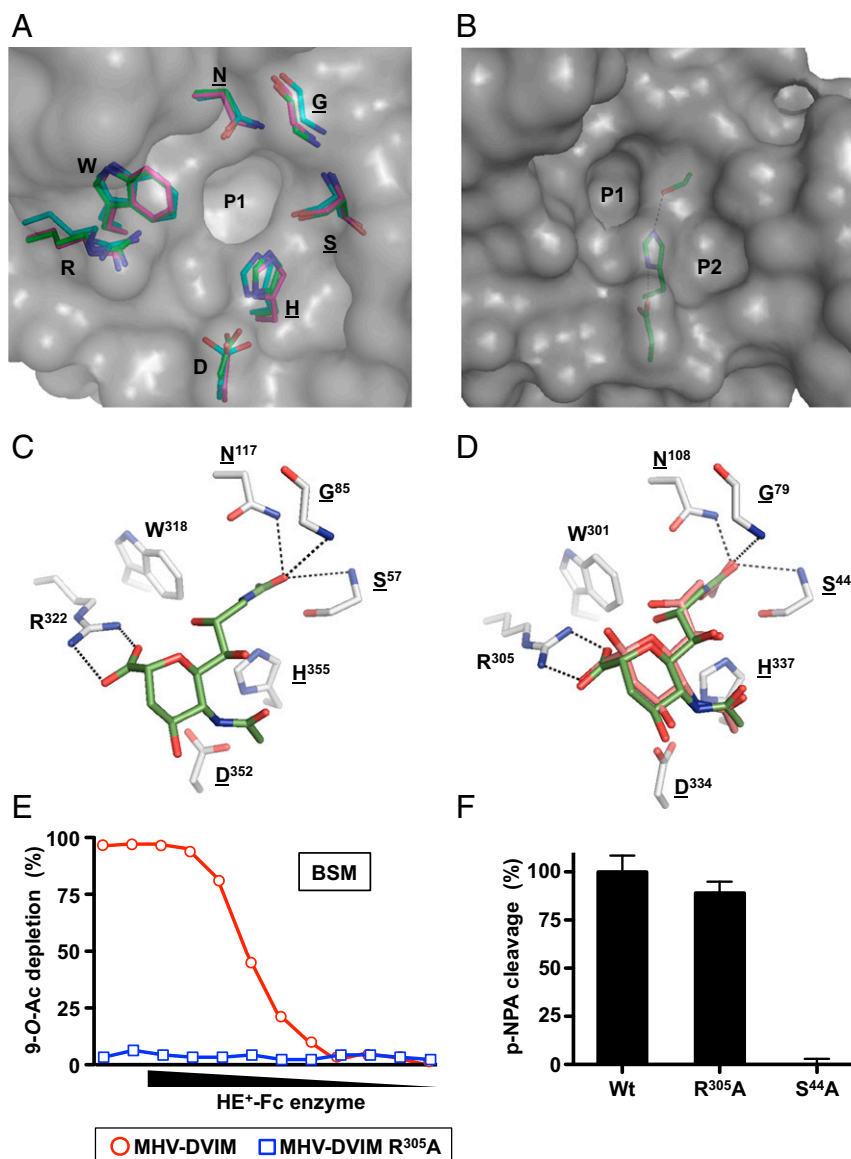


Fig. 2. (A) Superposition of residues lining the P1 pocket of Influenza C HEF (carbon atoms, cyan), MHV-DVIM HE (carbon atoms, green), and RCoV-NJ HE (carbon atoms, salmon). Surface representation is that of MHV-DVIM HE. Conserved residues within the SGNH family of hydrolases are underlined. (B) Surface representation of the catalytic center of MHV-DVIM HE with the P1 and P2 pockets indicated. The Ser-His-Asp catalytic triad is shown as sticks. (C) 9-N-Ac-Sia binding in the HEF catalytic site as observed in the crystal complex (18). Contacting amino acid side chains are shown in stick representation and colored by atom type (oxygen, red; nitrogen, blue; carbons, gray or green for amino acid side chains and 9-N-Ac-Sia, respectively). Oxyanion hole hydrogen bonds and the bidentate hydrogen bond interaction between Arg³⁰⁵ and the Sia carboxylate moiety are shown as black, dashed lines. (D) Model of 9-N-Ac-Sia binding in the MHV-DVIM HE catalytic site based on superposition with the HEF-inhibitor complex (carbon atoms, green) and on automated molecular docking (carbon atoms, salmon), represented as in Fig. 2C. (E) Catalytic activity of MHV-DVIM HE toward glycosidically bound 9-O-Ac-Sia is abrogated by substitution of Arg³⁰⁵ by Ala. Receptor destruction was assessed as in Fig. 1B. (F) Arg³⁰⁵Ala substitution in MHV-DVIM HE does not affect activity toward the synthetic substrate pNPA. Ser⁴⁴Ala is a catalytically inactive mutant. Enzymatic activity shown as percentage of wild-type activity.

binding topology of the substrate. As shown by the data, this is indeed what occurred (Fig. 3A). Compared with 9-N-Ac-Sia bound in the type I catalytic center of HEF (Fig. 2C) and to 9-O-Ac-Sia in the esterase site of MHV-DVIM HE as modeled by superposition or automated docking (Fig. 2D), the 4-N-Ac-Sia-substrate analog in the RCoV-NJ type II enzyme is rotated by 180° about the central Sia C2-C5 axis allowing the 4-N-acetyl moiety to be inserted into the P1 pocket while the 5-N-acyl remains in pocket P2 (Fig. 3A–C). Moreover, the substrate molecule is tilted by 20° such as to allow for sufficient space for the remaining sugar residues of the glycan chain to which the natural substrate, 4-O-Ac-Sia, would be attached.

As a corollary of the altered substrate topology, the catalytic site Arg, critical in type I HEs (20), can no longer interact with the Sia carboxylate. In accordance, substitution of RCoV HE Arg³⁰⁷ by Ala caused only a minor reduction in sialate-4-O-acetyl esterase activity (Fig. 3D and E). Thus, in type II HEs, the catalytic center Arg, although conserved, has become functionally redundant and is no longer essential for substrate binding.

Type II-Specific Amino Acid Substitutions Responsible for 4-O-Ac-Sia Substrate Specificity Revealed by Mutational Analysis. From the type II HE structure, it was not immediately evident how the shift in substrate specificity was achieved and how binding of the original

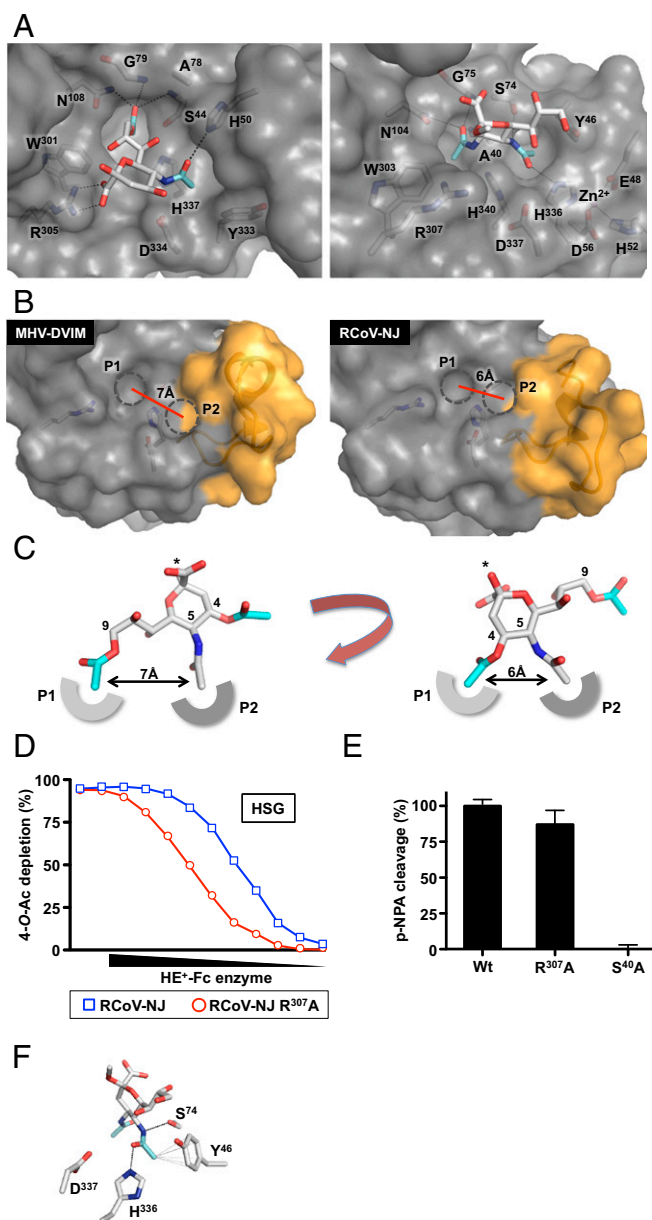


Fig. 3. (A) Surface representation of the MHV-DVIM HE (Left) and RCoV-NJ HE (Right) catalytic sites in complex with 9-*O*-Ac-Sia [docked with Autodock4 (55)] and 4-*N*-Ac-Sia (crystal complex), respectively. (B) Surface representation of the catalytic sites of MHV-DVIM HE (Left) and RCoV-NJ HE (Right). The active-site Ser⁴⁴ in MHV-DVIM HE already adopts the “active” rotamer observed in HEF (18, 39, 40); for RCoV-NJ HE crystallized as an inactive Ser-to-Ala mutant, a Ser side chain with active rotamer was introduced using COOT. The P1 and P2 pockets are highlighted by dashed circles; approximate distances between pockets, as measured from the centers, are indicated. (C) Binding topology of α Neu4,5,9Ac₃ in type I (Left) and type II (Right) esterases. The P1 and P2 pockets accommodating the *O*- and *N*-acetyl moieties are shown schematically. α Neu4,5,9Ac₃ is shown in stick representation and colored as in Fig. 2C. Asterisks indicate the position of the O₂ atom through which Sias are glycosidically linked. The distances between 5-*N*- and 9-*O*- or 4-*O*-Ac methyl groups are shown. (D) RCoV-NJ HE Arg³⁰⁷ is not essential for sialate-4-*O*-acetyltransferase activity. Ser⁴⁰Ala is a catalytically inactive mutant. Receptor destruction was assessed as in Fig. 1B. For a comparison with type I HEs, see Fig. 2E. (E) Arg³⁰⁷Ala substitution in RCoV-NJ HE does not affect activity toward the synthetic substrate pNPA. Enzymatic activity shown as percentage of wild-type activity. (F) Hydrogen bonding of the sialate-5-*N*-acyl carbonyl oxygen and amide nitrogen with RCoV-NJ HE Ser⁷⁴ and His³³⁶, respectively, as observed in the crystal complex, indicated as in Fig. 2C. Hydrophobic contacts between Tyr⁴⁶ and the Sia-5-*N*-acyl methyl group are shown as thin gray lines.

substrate is excluded. We therefore performed comprehensive comparative sequence analysis of all type I and II coronavirus HE esterase domains available in GenBank to identify consistent differences related to substrate specificity (for the nomenclature of protein segments, see Fig. S4; for an alignment of representative type I and type II HEs, see Fig. S1). Only a select number of such dissimilarities were noted involving three distinct elements (Fig. 4A and B). In segment β 2 α 3, proximal to the P2 pocket, there is a single type-specific amino acid difference: Ala in type I and Ser in all type II HEs. Far more prominent changes occurred in segment α 1 β 2, which comprises a surface-exposed disulfide loop (formed by Cys⁴⁴ and Cys⁶⁵ or Cys⁴⁸ and Cys⁶⁹ in RCoV-NJ HE and MHV-DVIM HE, respectively) with 16 out of 20 residues (80%) uniquely substituted in type II HEs. The other type II-specific differences are in segment β 16 α 6, entailing a single-residue insertion and the substitution of the orthologs of DVIM HE Val³³²-Tyr³³³ by Asp-Thr-His (Fig. 4A and B). Apparently, the changes that occurred in segments α 1 β 2 and β 16 α 6 are interrelated as they resulted, among others, in the creation of a novel metal-binding site, located near the active site and formed by the side chains of α 1 β 2 residues Glu⁴⁸, His⁵², Asp⁵⁶, and β 16 α 6 residue His³³⁶ (Fig. 4C). The presence of two negatively charged coordinating residues indicates that the site is occupied by a bivalent metal ion, which we identified as Zn²⁺ on the basis of (i) distances to coordinating amino acids (Tables S4 and S5), (ii) coordination by two acidic residues and two imidazole rings, and (iii) X-ray absorption data (Fig. S5). Apopos, loss of this metal ion, caused by the low pH crystallization conditions may well have caused the disorder of the catalytic domain in the published structure of MHV-S HE (21).

The introduction of the three type I elements of DVIM HE into the RCoV-NJ HE background resulted in an esterase with strict type I substrate specificity (Fig. 4D). The recombinant protein lost all enzymatic activity toward 4-*O*-Ac-Sias and, compared with the naturally occurring type I HE of MuCoV strain DVIM, even displayed a 12-fold higher sialate-9-*O*-acetyltransferase activity. We postulate that, in its esterase domain, the NJ/DVIM type I chimera is a facsimile, or at the least a close approximation, of the most recent common ancestor of the type II HEs (i.e., of the parental HE that still retained the original, type I specificity for 9-*O*-Ac-Sias). Departing from this perspective, we asked what the importance of the changes in the individual elements might be, and what the minimal requirements for the ancestral type I enzyme would have been to gain 4-*O*-acetyltransferase activity and to exclude the original (type I) substrate. To this end, we systematically placed back the type II elements into the type I chimera either individually or in combination (Fig. 4E). Separate reintroduction of the type II α 1 β 2 Cys loop or the β 16 α 6 segment did not result in renewed activity toward 4-*O*-Ac-Sia, but in either case, sialate-9-*O*-acetyltransferase activity was reduced significantly, i.e., by 92% (β 16 α 6) or even more, to below detection levels (α 1 β 2). Apparently, the type II-specific mutations in either of these two segments perturb the binding of the original type I substrate. Conversely, introduction of the single β 2 α 3 Ala⁷⁴Ser mutation in the type I chimera produced a hybrid enzyme that retained most of its sialate-9-*O*-acetyltransferase activity, but that now also accepted 4-*O*-Ac-Sia as a substrate. However, as the type II activity is only 25% of that of RCoV-NJ HE, the Ala-to-Ser substitution would not have sufficed to confer full 4-*O*-AE activity. Importantly, combinations of the Ala⁷⁴Ser substitution with either the type II β 16 α 6 segment or the α 1 β 2 Cys-loop did not have an additive effect on the cleavage of 4-*O*-Ac-Sias. Actually, the latter two segments are only functional in unison, as their combination gave an enzyme that cleaved 4-*O*-Ac-Sias, albeit very inefficiently (~5% of the activity of RCoV-NJ HE; Fig. 4E). Apparently, contribution of the β 16 α 6 and α 1 β 2 segments to type II esterase activity critically relies on formation of the novel intersegment metal-binding site. Indeed, single substitutions introduced into RCoV-NJ HE to disrupt metal binding (Glu⁴⁸Gln or Asp⁵⁶Asn) reduced

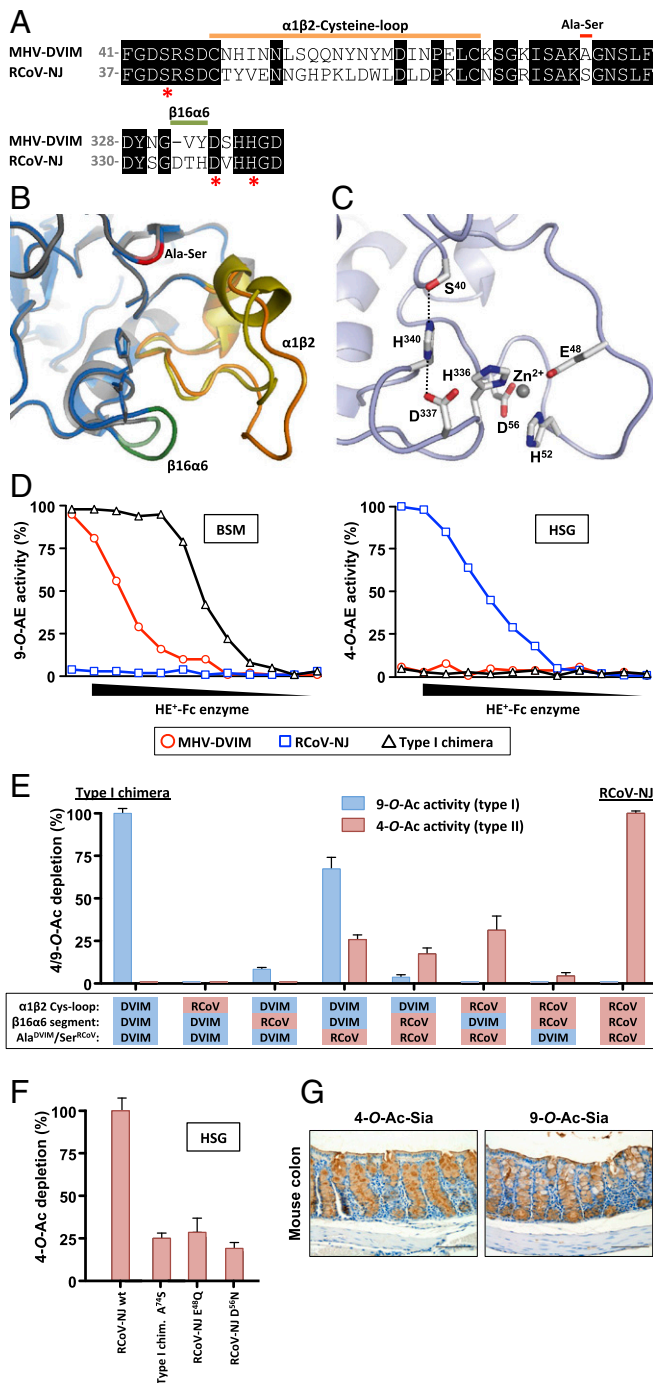


Fig. 4. (A) Partial sequence alignment of MHV-DVIM and RCoV-NJ HE, highlighting consistent differences between type I and type II HEs (Fig. S1). Aligned sequences, with residue numbering presented *Left and Right*, cover the $\alpha 1\beta 2$ -cysteine-loop, the $\beta 2\alpha 3$ segment (single Ala⁷⁸Ser substitution), and the $\beta 16\alpha 6$ segment. Catalytic residues (Ser, Asp, His) are marked with asterisks. (B) Overlay of cartoon representations of the active-site regions of MHV-DVIM HE (gray) and RCoV-NJ HE (blue). Side chains of catalytic triad residues are depicted as sticks. The three type I/II distinctive elements are colored as in A. (C) Cartoon representation of the novel metal-binding site near the RCoV-NJ HE active site, formed by Glu⁴⁸, His⁵², Asp⁵⁶, and His³³⁶. The catalytic triad is shown for reference. Side chains are depicted as sticks, the Zn²⁺ ion as a gray sphere. (D) A type II HE converted into a type I enzyme. An RCoV HE-based chimera with all three type I/II distinctive elements replaced by those of MHV-DVIM displays strict sialate 9-O-acetyltransferase activity. The enzyme activity of the recombinant protein ("Type I chimera") was compared with that of the parental proteins (MHV-DVIM and RCoV-NJ

sialate-4-*O*-acetyltransferase activity to 25%, i.e., the amount of type II activity that would be conferred by the Ala⁷⁴Ser substitution alone (Fig. 4*F*). From the combined findings, we conclude (*i*) that, during MuCoV evolution, the conversion of a type I HE into an enzyme with dual (type I and type II) specificity would have required a single Ala-to-Ser mutation; (*ii*) that, for this enzyme to have gained full 4-*O*-AE activity, the type II-specific changes in all three elements were necessary; and (*iii*) that the definitive shift in substrate specificity, i.e., the exclusion of the original type I substrate 9-*O*-Ac-Sia, must be attributed to the changes in the β 16 α 6 and α 1 β 2 segments.

Type II-Specific Substitutions: Structural Consequences for Substrate Binding. The consequences of the type II-specific amino acid substitutions become clear when they are considered in the context of the crystal structures of the type I and II HE esterase domains. The type II-specific mutations all affected the P2 pocket, virtually causing the pocket to shift by 2.8 Å along the ridge, formed by the catalytic triad, thus reducing the distance between the P1 and P2 pockets from 7 Å in MHV-DVIM HE [and all other orthomyxovirus, torovirus, and coronavirus type I HEs (18–20)] to 6 Å in RCoV-NJ HE (Fig. 3*B*). In reality, the original P2 pocket was lost and a new one created. Within the $\alpha 1\beta 2$ Cys loop, His⁵⁰ in DVIM HE was replaced by Tyr, the aromatic side chain of which is rotated by 20° (compared with that of DVIM HE His⁵⁰), opening a novel pocket of which it forms one side. Ser⁷⁴ of RCoV-NJ HE, the ortholog of which in DVIM HE is at the periphery of the catalytic center, now forms an adjacent side of the P2 pocket. His³³⁶ in the $\beta 16\alpha 6$ segment, replacing Tyr³³³ in DVIM HE, is pushed deeper into the catalytic center as a result of the type II-specific insertion of Asp³³⁴, and locked in position by metal coordination. Its side chain compared with that of DVIM Tyr³³³ is rotated by 35°, thus walling off the type II P2 pocket (Fig. 3*A*). The structure of the esterase–ligand complex provides an attractive explanation for the importance of the type II-specific changes in segment $\beta 16\alpha 6$ and the $\beta 2\alpha 3$ Ala-to-Ser substitution, as in RCoV-NJ HE, His³³⁶ and Ser⁷⁴ are ideally positioned for hydrogen bonding with the sialate-5-*N*-acyl carbonyl and -amide, respectively (Fig. 3*F*). Additionally, an important role is suggested for Tyr⁴⁶ in the $\alpha 1\beta 2$ Cys loop as it can form extensive hydrophobic contacts with the sialate-5-*N*-acyl methyl group (Fig. 3*F*). We propose that these new polar and hydrophobic interactions compensate for the loss of the Arg/sialate-carboxylate double-hydrogen bond interaction crucial to sialate-9-*O*-acetyl esterases and contribute to substrate binding in type II HEs by stabilizing 4-*O*-Ac-Sia in proper orientation in the catalytic center.

HE) on BSM (*Left*) and HSG (*Right*). Cleavage of 9-O- and 4-O-Ac-Sias was assessed as in Fig. 1*B*, but now starting at 10 ng/μL. (*E*) Contribution of the three type I/II distinctive elements to esterase activity and substrate specificity. The type I chimera was subjected to mutational analysis entailing systematic reintroduction of RCoV-NJ segments. Esterase activities of chimeric proteins toward 9-O-Ac- (blue bars) and 4-O-Ac-Sias (red bars) were determined in twofold dilution series as in Fig. 1*B*. Data are shown as percentages of specific esterase activity, calculated at 50% receptor depletion, relative to that of the type I chimera (for 9-O-Ac-Sia) or of wild-type RCoV-NJ HE (for 4-O-Ac-Sia). The error bars represent the SD over six measurements (two biological replicates, each of which performed in technical triplicates). (*F*) The type II esterase metal-binding site is required for full 4-O-AE activity. Note that disruption of metal binding by either Glu⁴⁸Gln or Asp⁵⁸Asn substitution reduces sialate-4-O-acetyl esterase activity by 75% (comparable to the amount of type II activity conferred by the Ala⁷⁴Ser substitution alone). Enzymatic activity measured as in Fig. 1*B* and presented as in Fig. 4*E*. (*G*) 4-O- and 9-O-Ac-Sias are abundantly expressed in the mouse colon. Paraffin-embedded mouse colon tissue sections were stained for 4-O-Ac-Sia with MHV-5 HE⁰-Fc, and for 9-O-Ac-Sia with PTOV-P4 HE⁰-Fc.

A Single Ala-to-Ser Amino Acid Substitution Was Key to the Emergence of Type II HEs. The shift in ligand/substrate preference in the MuCoV HE proteins required coevolution of two distinct domains, and, at first glance, the odds of this happening would seem remote. Clearly, the order in which the different events took place cannot be established, i.e., it is unknown whether a shift in lectin ligand specificity occurred first with a shift in esterase substrate specificity following suit or vice versa. In either scenario, however, the single substitution Ala-to-Ser in the $\beta 2\alpha 3$ segment would have been key. It is quite possible that the initial change occurred in the lectin domain through mutations that allowed chance low-affinity virion binding to 4-*O*-Ac-Sias. However, without an esterase domain to support catalysis-driven virion release from the new ligand, mutant viruses would have been fully dependent on the kinetics of the lectin–ligand binding equilibrium for reversibility of attachment. Thus, even with the lectin domain taking the lead, the novel receptor specificity might only have presented a viable evolutionary alternative for the parental type I binding, because of the fact that an enzyme with sialate-4-*O*-acetylerase activity could arise through a single amino acid substitution. In the reverse scenario, a single mutation in the esterase domain, resulting in a promiscuous enzyme that retained parental substrate specificity, but with the capacity to also cleave 4-*O*-Ac-Sias, might have set the stage for the changes in the lectin domain to occur, leading to a shift in ligand specificity. Be it as it may, at least the order of changes in the esterase domain itself can be understood. Conceivably, the single $\beta 2\alpha 3$ Ala-to-Ser substitution would have allowed further evolution toward optimal activity and substrate specificity of the enzyme. In this view, an inconspicuous point mutation opened the window of opportunity for the far more extensive, interdependent adaptations in the $\alpha 1\beta 2$ and $\beta 16\alpha 6$ segments to occur.

The Type II HE Receptor Switch Explained from the Stereochemistry of Protein–Carbohydrate Interactions. Specific recognition of sugars by proteins is subject to intricacies connected with carbohydrate structure and stereochemistry (41, 42). “Simple” monosaccharides like galactose and mannose offer few functional groups. Their hydroxyl moieties, constituting the principal binding partners in carbohydrate–protein interaction sites, are engaged in complex interaction networks involving direct or water-mediated hydrogen bonds and, often, metal ion coordination (41, 43). As such interactions commonly involve pairs of adjacent hydroxyls, the spatial arrangement of the two OH groups is imposed on the architecture of the CBS. With any such constellation not being unique to one particular monosaccharide, selection of the proper ligand and exclusion of closely related sugars requires additional specific interactions (43). On the flip side, this binding strategy confers a remarkable versatility such that with modest changes in protein structure through preservation of the geometry of the crucial hydrogen and coordinating bonds, the CBS can be adapted to fit alternative ligands and ligand topologies (41–48) (Fig. S6). Sias possess a large number of accessible functional groups (carboxylate, 5-*N*-acyl, the hydroxyls or substitutions thereof at ring atom C4 and at glycerol side chain atoms C7, C8, and C9), which, as argued by Neu et al. (3), should allow an “unparalleled number” of sugar–protein interactions. Although this is true, our findings described here and elsewhere (19–21) suggest that, for biomolecular recognition of 9- and 4-*O*-acetylated Sias, the same basic principles apply as were established for less complex monosaccharides. The shift in esterase substrate from 9- to 4-*O*-Ac-Sias was accomplished not through radical changes in protein architecture, but by altering ligand binding topology in the context of a largely conserved CBS. This was possible on account of (i) the fortuitous stereochemical similarity between 4-*O*- and 9-*O*-Ac-Sias with the 9-*O*- and 4-*O*-Ac moieties positioned at similar angles and roughly similar distances with respect to the central 5-*N*-acyl; and (ii) a recurring mechanism of protein binding to *O*-Ac-Sias, involving the recognition of pairs of identical functional groups (Ac-moieties)

based on shape complementarity, with the 5-*N*- and *O*-Ac-methyls docking into hydrophobic pockets astride of an intercalating aromatic amino acid side chain (19–21). The adaptations in the type II HE esterase are in fact analogous to those that took place in the corresponding type II lectin domain (21). In either case, the ancestral type I CBS was modified as to reduce the distance between *O*- and *N*-Ac docking sites to accommodate for the shorter distance between the sialate-4-*O*- and -5-*N*-acyl groups (6 Å, versus 7 Å for that between the sialate-9-*O*- and -5-*N*-acyls). In this sense, the reciprocal changes that occurred in the type II lectin and esterase domains to adjust ligand and substrate specificity present a singular case of convergent intramolecular coevolution.

HE Receptor Switching: Virus Evolution Driven by Sialoglycan Diversity Among Hosts and Tissues? The mere occurrence of the type II MuCoV biotype implies that the shift to using 4-*O*-Ac-Sias for virion attachment resulted in a gain in viral fitness. Although we now understand in structural terms how the transition in ligand/substrate specificity occurred, it remains an open question what biological conditions triggered the emergence of the type II HEs and favored their selection. Both 9- and 4-*O*-Ac-Sias are abundant in the murine gastrointestinal tract, particularly in the colon (Fig. 4G) (8), and the cocirculation of type I and II MuCoVs in nature indicates that in principle either type of Sia can serve as attachment factor. There may be differences, however, in expression levels and/or in tissue and cell distribution between 9- and 4-*O*-Ac-Sias—subtle or less subtle—that so far have gone unnoticed, and that were yet of decisive importance. Saliently, of 27 strains in the species *Murine coronavirus* identified so far, only three (DVIM, MI, and -2) possess a type I HE. It is tempting to speculate that type I MuCoVs represent an ancestral biotype that is gradually being replaced by type II. However, our knowledge of MuCoV diversity in nature is limited and restricted to a relatively small number of laboratory isolates mostly from mice (*Mus musculus domesticus*) and rats (*Rattus norvegicus*) kept in animal facilities. We have little to no understanding of the complexity and interspecies diversity of the sialomes in naturally occurring murids or in other mammals for that matter. It is in the unraveling of how such factors might direct virus evolution that a next challenge lies.

Materials and Methods

Expression and Purification of CoV HEs. Human codon-optimized sequences for the HE ectodomains of RCoV-NJ (residues 22–400) and MHV-DVIM (residues 24–395) were cloned in expression plasmid pCD5-T-Fc (19). The resulting constructs code for chimeric HE proteins that (i) are provided with a CD5 signal peptide, and, C-terminally, with a thrombin cleavage site and the human IgG1 Fc domain, and that (ii) are either enzymatically active (HE-Fc) or rendered inactive through catalytic Ser-to-Ala substitution (HE⁰-Fc). Site-specific mutations were introduced by Q5 PCR mutagenesis (New England Biolabs). For receptor destruction esterase assays, HEs were produced by transient expression in HEK293T cells and purified from cell culture supernatants by protein A-affinity chromatography and low-pH elution as described (19). For crystallization, HEs were transiently expressed in HEK293 GnTI(–) cells (49), and the ectodomains were purified by protein A-affinity chromatography and on-the-beads thrombin cleavage as described (19). Purified HEs were concentrated to 5–10 mg/mL, and, in the case of RCoV-NJ, deglycosylated by the addition of 1 MU/mL EndoH_F (New England Biolabs), and incubated for 1 h at room temperature before the setup of crystallization experiments.

Crystallization and X-Ray Data Collection. MHV-DVIM HE crystals with P2₁2₁2₁ space group were grown at 20 °C using sitting-drop vapor diffusion against a well solution containing 0.1 M Tris-HCl, pH 8.0, 0.05 M NaF, 16% (wt/vol) PEG3350, and 10% (vol/vol) glycerol. RCoV-NJ HE⁰ crystals with C22₁ space group grew against two different well solutions: 0.1 M Bis-Tris propane, pH 7.5, 0.2 M NaF, and 20% (wt/vol) PEG3350; and 0.1 M Hepes, pH 7.5, 0.2 M NaCl, and 20% (wt/vol) PEG3000. The structure of RCoV-NJ HE without ligand was obtained from the first condition, and the structure of RCoV-NJ HE in complex with receptor analog was obtained from crystals grown in the latter condition. These latter crystals were, before flash-freezing, soaked for 10 min at 4 °C in cryoprotectant containing 100 mM 4,5-di-*N*-acetylneuraminic acid α -methylglycoside (for the synthesis of this compound, see *SI Materials and Methods* and

Fig. S7. Crystals were cryoprotected in well solution containing 20% (RCov-NJ) or 12.5% (MHV-DVIM) (vol/vol) glycerol before flash-freezing in liquid nitrogen. Diffraction data of MHV-DVIM was integrated with Eval15 (50) and diffraction data of RCov-NJ was integrated with Mosflm (51). Integrated diffraction data were further processed using the CCP4 package (52). The structures of RCov-NJ HE and MHV-DVIM HE were solved by molecular replacement using the HE structure from MHV-S [PDB ID code 4C7L (21)] and BCoV-Mebus [PDB ID code 3CL5 (19)] as search models, respectively. Models were refined using REFMAC (53) alternated with manual model improvement using COOT (54). Refinement procedures included TLS refinement using either one (RCov-NJ HE) or three TLS groups per molecule (MHV-DVIM HE). For RCov-NJ HE⁰ free, R_{work} and R_{free} had final values of 23.2% and 27.8%. For RCov-NJ HE⁰ complexed with 4,5-di-*N*-Ac-Sia, R_{work} and R_{free} had final values of 18.5% and 20.3%. For MHV-DVIM HE, these values were 19.9% and 22.6%, respectively. Statistics of data processing and refinement are listed in Table 1.

X-Ray Fluorescence Measurements. X-ray absorption spectra were recorded from RCov HE crystals on European Synchrotron Radiation Facility (ESRF) beamline ID29 in fluorescence mode using a Rontec Xflash X-ray fluorescence detector. The X-ray energy was scanned around the Zn K-edge ($\lambda = 1.28 \text{ \AA}$; energy = 9,668 eV).

Molecular Docking. Molecular docking of 9-*O*- and 4-*O*-Ac-Sia in the crystal structures of MHV-DVIM HE and RCov-NJ HE, respectively, was performed with AutoDock4 (55). The Sia molecules used for docking were extracted from BCoV HE (PDB ID code 3CL5; for 9-*O*-Ac-Sia) and from MHV-S HE (PDB ID code 4C7W; for 4-*O*-Ac-Sia). Ligand files were processed with AutoDockTools. During docking, the protein was considered to be rigid. This assumption is justified by the observation that binding of substrate analogs in the crystal structures of HEF and RCov-NJ HE does not induce conformational changes, except that in HEF, a rotation of the active-site Ser side chain was observed (39). Active-site Ser⁴⁴ in MHV-DVIM HE already adopts the “active” rotamer observed in HEF; for RCov-NJ HE, which was crystallized as an inactive Ser-to-Ala mutant, a Ser side chain with active rotamer was introduced using COOT. We used an inverted Gaussian function (50-Å half-width; 15-kJ energy at infinity) to restrain the *O*-acetyl carbonyl oxygen in the oxyanion hole at a position occupied by a water molecule in the respective crystal structures. The carbonyl oxygen must be located close to this position to enable charge stabilization of the negatively charged tetrahedral reaction intermediate, which is a critical step in the well-established reaction mechanism (39, 40). To reproduce the observed binding modes of substrate (analogs) in the active site of HEF and the lectin domains of RCov-NJ HE and BCoV HE, it proved necessary to constrain the torsion angles internal to the glycerol moiety to values observed in the RCov-NJ HE and BCoV-Mebus HE complexes. These values are very similar in both HE complexes as well as in numerous other Sia-protein complexes in the PDB. The initial ligand conformation was randomly assigned and 10 docking runs were performed. The method was validated by docking 9-*O*-Ac-Sia in the MHV-DVIM HE structure, which gave a mode of binding essentially identical to that of the substrate molecule from HEF superimposed on the MHV-DVIM HE structure (Fig. 2 C and D), and, by docking 4-*O*-Ac-Sia in the RCov-NJ HE structure, which gave an identical mode of binding for the 10 lowest energy solutions, which were es-

entially identical to that observed for the crystal complex with 4-*N*-Ac-Sia (Fig. 3A and Fig. S8).

Receptor Destruction Esterase Assay. The enzymatic activity of MHV-DVIM and RCov-NJ HE toward *O*-acetylated Sias was measured as described (21). Briefly, MaxiSorp 96-well plates (Nunc), coated for 16 h at 4 °C with 100 μL of HSGs (undiluted; TCS Biosciences) or BSM (1 $\mu\text{g/mL}$; Sigma), were treated with twofold serial dilutions of enzymatically active HE (starting at 100 ng/ μL in PBS, unless stated otherwise in the figure legend) for 1 h at 37 °C. Depletion of *O*-Ac-Sia was determined by solid-phase lectin-binding assay (8, 21) with lectin concentrations fixed at half-maximal binding (MHV-S HE⁰-Fc, 5 $\mu\text{g/mL}$, for 4-*O*-Ac-Sia; PTOV-P4 HE⁰-Fc, 1 $\mu\text{g/mL}$, for 9-*O*-Ac-Sia). Incubation was for 1 h at 37 °C; unbound lectin was removed by washing three times, after which bound lectin was detected using an HRP-conjugated goat anti-human IgG antiserum (Southern Biotech) and TMB Super Slow One Component HRP Microwell Substrate (BioFX) according to the instructions. The staining reaction was terminated by addition of 12.5% (vol/vol) H_2SO_4 and the optical density was measured at 450 nm. Graphs were constructed using GraphPad (GraphPad Software). All experiments were repeated as biological replicates at least two times and each time in technical triplicate, yielding identical results.

pNPA Assay. 4-Nitrophenyl acetate (pNPA) yields a chromogenic *p*-nitrophenolate anion (pNP) upon hydrolysis, which can be monitored at 405 nm. HE-Fc esterase activity toward pNPA was measured essentially as described (56). Briefly, 50 ng of HE was incubated with 1 mM pNPA in PBS and the amount of pNP was determined spectrophotometrically at 405 nm every 20 s for 15 min. Specific activity was defined as product yield/mass of enzyme (micromolar pNP per microgram of HE) and subsequently expressed as a percentage of wild-type HE activity.

O-Ac-Sia Expression in Mouse Colon. Tissue stainings were performed as described (8). In short, paraffin-embedded colon sections (Genta; AMS541) were dewaxed in xylene and rehydrated. 4- and 9-*O*-Ac-Sias were detected by incubating with MHV-S and PTOV-P4 HE⁰-mFc virolectins, respectively, and subsequently incubated with biotinylated goat- α -mouse IgG antibodies (Sigma-Aldrich; 1:250), with avidin-biotin HRP complex (ABC-PO staining kit; Thermo Scientific), and with 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich). Counterstaining was done with Mayer's hematoxylin; tissue sections were embedded in Eukitt mounting medium (Fluka) and examined by standard light microscopy.

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