# Sialic acids in nonenveloped virus infections

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# 1. Sialic acids as viral receptors—one term, many functions

Mammalian cells are covered in glycosylated proteins and lipids, and with the notable exception of glycosaminoglycans, sialic acids are the most common outermost units of glycosylated plasma membrane constituents. It is hence no surprise that sialic acids act as receptors for several viruses and virus classes, yet the specific roles which sialic acid and other virus receptors fulfill during viral entry vary from virus to virus and from cell type to cell type.

The most commonly used definition of a viral receptor is a plasma membrane constituent that binds to an incoming virus in a specific manner and enables subsequent cell entry steps that ultimately lead to productive infection, i.e., production of progeny virus. The fact that individual cellsurface components are differentially expressed on different cells types, in combination with the specificity of many virus-receptor interactions, is regarded as a major determinant of viral tropism, i.e., the restriction of a given viral infection to a limited set of host cell types. But such entry receptors can be accompanied by other types of receptors which mediate processes that are also important or even essential for productive entry.<sup>1</sup> Processes mediated by additional coreceptors include initial attachment, triggering of structural changes on the virus side prior to binding of the entry receptor, upregulation of signaling pathways in the target cell that aid entry, and recruitment of entry receptors via changes of the local plasma membrane composition. Attachment factors or attachment receptors interact only transiently with virions and do not trigger internalization but prolong virus residence time in proximity to the internalizing postattachment receptor. This is a relatively common role for sialic acid: many sialic acid-dependent viruses require a specific cell-surface protein for entry in addition to a sialylated attachment receptor (Table 1).<sup>26,27</sup> Some viruses attach to several types of receptors, each of which is essential, e.g., two types of attachment proteins. A set of receptors often binds in a specific sequential manner where the first interaction primes an incoming virus structurally for interaction with the actual entry receptor, e.g., by introducing a conformational change in the viral-attachment protein. In some cases, such a structural change enables enzymatic processing of the virus before interaction with the entry receptor and actual uptake take place.<sup>28</sup> Some viruses can use alternative receptors that all allow for productive infection, for instance, on different cell types. On the other hand, not all cell-surface molecules that mediate uptake guide the virions to cellular pathways through which productive infection occurs, giving rise to the terms "decoy" and "pseudoreceptors." This is, for example, the case for the GD3 ganglioside and murine polyomavirus (mPyV).<sup>29</sup> Gangliosides are membrane glycosphingolipids containing sialic acid residue(s). Last but not least, internalization can be preceded or accompanied by interactions that initiate signaling pathways that assist in entry, for example, by facilitating transport across the cortex barrier.<sup>30</sup>

Table 1 Sialic Acid-	Dependent Viruses Disc	cussed in Detail in this Work	Biological		
Virus	Natural Tropism and Natural Host	Sialic Acid-Bearing Receptor	Role of Sialylated Receptor	Other Documented Receptor(s)	Known Entry Pathway(s)
Simian virus 40 (SV40)	Renal (rhesus macaque)	GM1 ganglioside <sup>2</sup>	Entry receptor	None	Cav-1 <sup>a</sup> -dependent-and Cav-1-independent cholesterol-dependent endocytosis (alternative pathways) <sup>3,4</sup>
Murine polyomavirus (mPyV)	Various tissues (newborn mice)	GD1a/GT1b gangliosides <sup>2</sup> GT1a <sup>5</sup>	Entry receptor	None	Cav-1-dependent monopinocytosis <sup>6,7</sup>
BK polyomavirus (BKPyV)	Renal (human)	GD1b/GT1b gangliosides <sup>8</sup>	Entry receptor	GAGs as facultative attachment and entry receptors <sup>9</sup>	Cav-1-dependent and Cav-1- and clathrin- independent cholesterol-dependent pathway <sup>10</sup>
JC polyomavirus (JCPyV)	Renal, neuronal (human)	N-linked LSTc on glycoprotein <sup>11</sup>	Attachment receptor	Serotonin receptor as entry receptor <sup>12</sup> ; GAGs as facultative attachment and entry receptors <sup>9</sup>	Clathrin-mediated endocytosis <sup>13</sup>
Merkel cell polyomavirus (MCPyV)	Skin (human)	GT1b ganglioside <sup>14</sup> GM3 ganglioside <sup>15</sup>	Entry receptor	Sulfated glycosaminoglycans as attachment receptors <sup>15</sup>	Unknown
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Virus	Natural Tropism and Natural Host	Sialic Acid-Bearing Receptor	Biological Role of Sialylated Receptor	Other Documented Receptor(s)	Known Entry Pathway(s)
Human adenovirus (Ad)	Ocular (human, Ad37) Enteric (human, Ad52)	O-linked GD1a on glycoprotein (D-type, Ad37) <sup>16</sup> Polysialic acid (G-type, Ad52) <sup>17</sup>	Attachment receptor	Ad37: none Ad52: CAR <sup>17</sup>	Clathrin-mediated endocytosis <sup>18</sup>
Reovirus	Upper respiratory and gastrointestinal tracts, neuronal (newborn mice)	GM2 (for T1L) Sialylated glycans (T3D)	Adhesion strengthening	JAM-A (all reoviruses) <sup>19–21</sup> ; Nogo receptor NgR1 on neurons for type 1 <sup>22</sup>	Clathrin dependent <sup>23,24</sup>
CVA24v	Ocular (human)	O-linked sialic acid on glycoprotein	Attachment receptor	Second receptor likely <sup>25</sup>	Not known
<sup>a</sup> Cav-1, caveolin-1.					

Table 1 Sialic Acid-Dependent Viruses Discussed in Detail in this Work—cont'd

The entry mechanism of sialic acid-dependent viruses also varies. The small, nonenveloped viruses discussed in more detail in this review use clathrin-mediated endocytosis, caveolae-dependent endocytosis, and/or cholesterol-dependent pathways (Table 1). Of these, clathrin-mediated entry is the best studied pathway since clathrin-mediated endocytosis is generally one of the main cellular endocytosis pathways. Caveolae are lipid rafts primarily formed by the protein caveolin-1, which, promoted by local enrichment of cholesterol, assembles to form microdomains and endocytic invaginations. Since both clathrin and caveolin-1 are cellular proteins whose inherent function is the formation of endocytotic vesicles in the absence of viral infection, preformed microdomains enriched in these proteins exist in healthy cells and can be exploited by viruses. However, it appears that virus-receptor interactions can also precede targeted recruitment of these endocytotic proteins, creating novel endocytotic vesicles around receptor-bound viruses.<sup>1</sup> For a detailed description of clathrinand caveolae-dependent endocytosis, see ref. 3. Some viruses use neither clathrin nor caveolin for cell entry, instead relying on a range of other strategies that include cholesterol-mediated endocytosis via local membrane deformation (Table 1).<sup>3</sup>

### 2. Identification of sialic acid as a determinant of infection

#### 2.1 Hemagglutination assays

The two most common forms of sialic acid targeted by human and mammalian viruses are 5-*N*-acetylneuraminic acid (Neu5Ac) and 5-*N*glycolylneuraminic acid (Neu5Gc). The latter structure occurs in many mammals in addition to Neu5Ac, but humans and some mammals cannot synthesize it due to *CMAH* gene defects that result in the loss of the hydroxylase enzyme that converts Neu5Ac to Neu5Gc (Fig. 1). A first, relatively simple experiment that can serve to test involvement of any type of sialic acid in viral infection is the hemagglutination assay, one of the historically oldest virological experiments. Red blood cells, which are rich in sialic acid, are agglutinated by sialic acid-binding viruses, a phenomenon that was first observed by chance in influenza-infected chicken embryos and quickly turned into a technique to measure virus stock concentrations—for which the assay still serves today.<sup>31,32</sup> Hemagglutination assays are usually conducted with serial dilutions of virus stocks and often require several types of erythrocytes (i.e., from different animals) as many viruses agglutinate only certain erythrocytes. This phenomenon



**Fig. 1** Chemical structures of *N*-acetylneuraminic acid (Neu5Ac, *left*) and *N*-glycolylneuraminic acid (Neu5Gc, *right*).

may be explained by the varying amounts and types of sialic acid expressed on different types of erythrocytes and by the receptor specificity on the virus side, but is, of course, not necessarily consistent with the virus host range. Murine polyomavirus (mPyV), for instance, was identified as a sialic acid-binding virus via agglutination of hamster, guinea pig, and human erythrocytes,<sup>33</sup> and the human tumorigenic Merkel cell polyomavirus (MCPyV) agglutinates sheep but not human erythrocytes.<sup>15</sup> Apart from this host range-independent species specificity, the assay is not conclusive with regard to exactly which sialylated plasma membrane components are bound because our knowledge of the nature and amount of sialylated glycans on erythrocytes is fragmented. It can, however, point to differences in receptor usage between related viruses. For instance, all three mammalian reovirus prototype strains (T1L, T2J, and T3D) agglutinate human erythrocytes, but only the T3D strain agglutinates bovine erythrocytes,<sup>34,35</sup> a hint to the indeed differing sialylated receptor specificities of reovirus strains (see Section 4.1). Thus, the hemagglutination assay is an early tool in the sialic acid experimental "pipeline," helping to assess whether any type of sialic acid may assist in infection. If hemagglutination is observed, a role of sialic acid in virus attachment is likely (and can be substantiated with neuraminidase treatment as a control experiment), but additional receptors that are not sampled in the hemagglutination experiment may be required, and the exact biochemical identity of the sialylated (co)receptor remains to be elucidated. However, red blood cell membranes can also serve in later steps of receptor identification, despite the fact that erythrocytes are not usually virus target cells. For instance, treatment of erythrocyte membrane preparations with protease, combined with virus binding and sucrose gradient floatation assays, were the first steps toward identification of gangliosides as coreceptors for both mPyV and simian virus 40 (SV40).<sup>2</sup>

#### 2.2 Neuraminidase treatment

Further evidence for a role of sialic acids during viral infection is often achieved by its trimming from cultured cell surfaces and the resulting

diminished virus binding and/or infection. Reduction or near-complete removal of sialic acids from the plasma membrane can be achieved via incubation with neuraminidase, temporary knockdown of cellular pathways that orchestrate sialic acid presentation, or the use of cell lines that are permanently sialic acid deficient. Broad-range neuraminidases cleave, in principle, terminal sialic acid caps off different types of glycoconjugates and different types of glycosidic linkages, i.e.,  $\alpha 2$ -3,  $\alpha 2$ -6, and  $\alpha 2$ -8-linked sialic acid, albeit with varying kinetics. Unfortunately, some sialylated biomolecules are fairly resistant to neuraminidase treatment. The widely used Vibrio cholerae neuraminidase was, for instance, repeatedly shown not to modify the GM1 ganglioside (Fig. 2), a (co)receptor for SV40 and several bacterial toxins.<sup>36,37</sup> Generally, only 60%–80% of sialic acid content seems removable from different cell lines with V. cholerae neuraminidase.<sup>38</sup> GM1 is, however, cleavable with neuraminidase from Arthrobacter ureafaciens, and with certain buffer additives, by neuraminidase from Clostridium perfringens.<sup>39</sup> Because it is difficult to assess how complete and broad sialic acid removal through neuraminidase treatment is, unambiguous results may be best achieved through combinations with complementary experiments and implementation of careful controls. For mPyV, for example, an elegant complementation of neuraminidase treatment of cultured rodent cells (which results in decreased infectivity) was conducted by subsequent restoration of linkage-specific sialylation. To this end, activated sialic acid and a recombinant  $\alpha$ 2-3-specific glycosyltransferase were used, indeed restoring infectivity and lending more weight to the sialylated receptor hypothesis while also suggesting a specific sialic acid linkage requirement of mPyV in the same experiment.<sup>40</sup> In some cases, differential use of neuraminidases with differing specificities may help to elucidate the sialic acid linkage type of a specific receptor, as shown for the human JC polyomavirus (JCPyV).<sup>41</sup>

#### 2.3 Inhibition of glycosylation

Temporary knockdowns of sialic acid synthesis or presentation can be achieved by chemical inhibitors or siRNA treatment. A number of wellcharacterized inhibitors exist. A widely used inhibitor of protein Nglycosylation is the antibiotic tunicamycin, a UDP-GlcNAc analogue that inhibits the first step of N-glycan chain synthesis and can thus help to assess if the virus receptor under investigation is a glycoprotein of the N-glycosylated type, the O-glycosylated type, or a glycolipid.<sup>41</sup> Interestingly, the compound also inhibits formation of enveloped viruses, probably because it interferes with production of stable envelope glycoproteins.<sup>42</sup>

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Fig. 2 Schematic structures of sialylated oligosaccharides discussed in the text.

The concentrations required for inhibition of N-glycosylation and the extent of side effects on other cellular functions vary between cell lines and depend on additional experimental parameters such as the culture medium. It is self-evident that inhibition of glycosylation can have drastic side effects on protein synthesis and transport.<sup>42</sup> In fact, tunicamycin is reported to arrest cells in the G1-phase of the cell cycle as a consequence of triggering the unfolded protein response signaling pathway,<sup>43</sup> which might influence progeny virus production by additional mechanisms independent of cellular uptake, as many viruses exploit the unfolded protein response. Additionally, because the glycosyltransferases in the ER and Golgi apparatus are themselves glycosylated, it is feasible that O-glycosylation and glycolipid production can also be affected by tunicamycin, i.e., the inhibitor might not exclusively reduce N-glycosylation. For instance, synthesis of the GM1 and GM2 gangliosides, (co)receptors for SV40 and reovirus T1L, respectively (Fig. 2), is inhibited by tunicamycin at least in certain cell lines.<sup>44,45</sup> Nevertheless, this antibiotic is a standard tool in the identification of glycosylated virus receptors.

The O-glycosylation counterpart of tunicamycin is benzyl 2-acetamido-2-deoxy- $\alpha$ -D-galactopyranoside (benzyl-N-acetyl- $\alpha$ -galactosaminide; benzyl- $\alpha$ -D-GalNAc), a potent inhibitor of mucin biosynthesis.<sup>46</sup> It was successfully used, for instance, for adenovirus 52 (Ad52), a virus with unknown tissue tropism and a rare polysialic acid preference.<sup>47</sup> Although precursors of O-glycosylation accumulate also in benzyl- $\alpha$ -D-GalNAc-treated cells, the inhibitor seems to be more specific in terms of affecting one type of glycosylation, only, probably because glycosyltransferases tend to carry more N- than O-glycosylation.<sup>46</sup>

#### 2.4 Sialic acid-deficient cell lines

There are numerous glycosylation-deficient cell lines for applications in virology, many of which are based on Chinese hamster ovary (CHO) cell clones, which were isolated by selection for resistance to plant lectin cytotoxicity (for a comprehensive review of CHO-derived cell-line glyco-sylation, see ref. 48). CHO-Lec2 cells have a much reduced capability of transporting activated sialic acid to the Golgi apparatus, leading to an "asialo" cell surface with a sialic acid reduction of 90% or more.<sup>48–50</sup> Wild-type CHO cells cannot produce complex glycolipids other than the simplest ganglioside and precursor of complex ganglioside synthesis, GM3 (Fig. 2). Thus, a combination of both cell lines or restoration of

GM3 synthesis in CHO-Lec2 cells via introduction of an intact CMP-sialic acid transporter *SLC35A1* gene can help identify GM3 or a noncomplex sialylated glycoprotein as a virus–receptor candidate.<sup>15</sup> Treatment with proteases may then be used to distinguish between the ganglioside and a glycoprotein receptor—a strategy that was, for example, used to identify the adenovirus 37 (Ad37) receptor as a sialylated glycoprotein.<sup>51</sup> Wild-type CHO and HeLa cells carry comparable densities of sialic acid on the plasma membrane.<sup>38</sup>

Many immortalized cell lines display altered glycosylation, a general hallmark of cancer. For instance, rat C6 glioma cells are also unable to produce complex gangliosides and have served, upon supplementation with specific complex gangliosides, to identify the branched gangliosides GD1b/GT1b and GM1 as mPyV and SV40 (co)receptor, respectively (Fig. 2).<sup>2</sup>

Since sialic acid-deficient cell lines display permanently reduced sialic acid content, they may appear as a more reliable experimental approach when compared to enzymatic or inhibitor-of-glycosylation treatments. However, the use of nonphysiological cell lines that are often not productively infected, but display convenient sialic acid deficiencies, has its own caveats since viruses can also be taken up by nonpermissive cell lines and subsequently routed to noninfective cellular pathways. MPyV, for instance, is internalized in the absence of gangliosides but requires the latter for productive infection.<sup>29</sup> In experiments that monitor uptake rather than productive infection, i.e., fluorescence microscopy and thin-section TEM, viruses may appear to have been successfully internalized, while they may, in fact, be trapped in dead-end pathways. This is a particular concern (not just in the context of sialic acid) for viruses whose cellular tropism, i.e., in which cell type the virus replicates in a physiological environment, is not yet known or for which productively infected cell lines are difficult to cultivate in vitro. For these viruses, cell-culture experiments have to be evaluated with caution since results from cell lines that do not support stable infection can be misleading. Generally, late steps of the virus life cycle should be monitored where possible, e.g., by production of progeny virus or expression of viral proteins like the polyomavirus large T-antigen. In the case of DNA viruses, transduction can be used as a hallmark of productive entry using pseudovirions (PsVs), i.e., particles composed of the intact viral capsid but loaded with a reporter gene plasmid rather than viral DNA.<sup>15</sup> If reporter genes are chosen that encode fluorescent proteins, the delivery of viral DNA to the nucleus can be monitored by fluorescence microscopy, i.e., entry and uncoating of PsVs serve as readouts rather than internalization.<sup>52</sup>

As mentioned in the foregoing, in experiments that are complementary to using sialic acid-deficient or -depleted cell lines, cultured cells can also be altered to present *increased* amounts of sialic acid on the plasma membrane, e.g., via restoration of sialic acid biosynthesis/transport or overexpression of candidate glycoproteins. In the case of glycolipid receptors, direct supplementation of cells with receptor candidates is possible as glycolipids are readily incorporated into the plasma membrane. In favorable cases, this procedure may allow for a comprehensive characterization of the receptor profile. As an example for a ganglioside-dependent virus, infection of a permissive cell line by human BKPyV can be increased by b-series gangliosides GD3, GD2, GD1b, and GT1b, but not by the a-series ganglioside GM1 (Fig. 2). These observations are in line with a Neu5Ac $\alpha$ 2-8Neu5Ac epitope bound in a crystal structure of the BKPyV major capsid protein (see Section 4.2)<sup>53</sup> and previous reports that BKPyV cell attachment is resistant to proteinase K but not to C. perfringens neuraminidase.<sup>8</sup> In fact, supplementation with b-series gangliosides can even turn a nonpermissive cell line into a permissive cell line, as evidenced by BKPyV T-antigen expression in LNCaP cell lines upon ganglioside supplementation,<sup>8</sup> highlighting the important role of plasma membrane constituents for tissue tropism.

In the case of lipid-linked, sialylated carbohydrates such as gangliosides, the functional relevance of an oligosaccharide structure can also be directly tested using ganglioside supplementation assays. As gangliosides are synthesized via a common pathway that requires the simplest ganglioside, GM3, cells lacking the GM3 synthase are unable to produce gangliosides, and their cell membranes are, therefore, devoid of all gangliosides. These cells can then be supplemented with a specific ganglioside to test whether this supplementation allows a virus to bind and enter the cell. In this manner, the functionality of a receptor candidate can be directly evaluated and compared against other related structures. The ganglioside receptor for SV40, GM1, was, for example, first identified using this approach.<sup>2</sup>

#### 2.5 Discrepancies between in vivo and in vitro "receptors"

Discrepancies between in vitro and in vivo studies are sometimes explained by considering the different roles that "receptors" can adopt (see Section 1) and the fact that not all interaction partners at the plasma membrane may be directly involved in internalization. For polyomaviruses (PyVs) in particular, it is emerging that cell attachment, uptake, and routing to infectious pathways (retrograde transport to the ER, that is) may be mediated by different plasma membrane components. In this regard, it is possible that nonsialylated components of the plasma membrane mediate attachment, while specific sialylated receptors are necessary to route internalized virions to the ER. This phenomenon is best described for MCPyV, which requires glycosaminoglycans (GAGs) as attachment receptors, but the noncomplex ganglioside GM3 for transduction.<sup>15</sup> Since GAGs have been shown to also interact with other PyVs, including examples for which receptors for productive infection have already been identified as sialylated compounds, it is possible that such a distinction may also emerge for other members of the genus Polyomaviridae.<sup>9</sup> Alternatively, some members of the family may simply exhibit a certain promiscuity with respect to receptor usage. Another aspect, which is often not probed for in cell-culture experiments, may be the modulation of host-cell signaling and immune response. Such processes can be stimulated by a virus binding to additional plasma membrane components that may then not turn out as entry receptors in, for example, mice models, but could nevertheless be identified in in vitro studies such as the micro glycan arrays. Alternatively, interaction partners that are identified in in vitro approaches could act as decoy or pseudo-receptors, i.e., some form of host defense, as was pointed out for GD3 and mPyV.<sup>29</sup> Lastly, a role of the microbiota, an emerging theme in enteric virus infection, could also lead to apparently conflicting observations between different sets of experiments, which is again a point that is, at the moment, difficult to assess experimentally (see Section 5).

#### 2.6 Other experiments

Another type of complementary experiment makes use of specific lectins as competitors. Here, the  $\alpha$ 2-3-linked sialic acid-specific *M. amurensis* (MAA) and the  $\alpha$ 2-6-linked sialic acid-specific *S. nigra* (SNA) are often used.<sup>51</sup> Both lectins are also employed to assess the completeness of neuraminidase treatment of cultured cells. Lectin binding is, however, often not exclusively mediated by specific interactions; thus, again, this set of experiments should be interpreted with reservations.

Enzymes other than neuraminidases, such as proteases, can be used to distinguish between glycoproteins and glycolipids, and PNGase can help to distinguish between N- and O-linked glycoproteins.<sup>41</sup>

Knockout mice with defects in specific sialylated glycan pathways can sometimes be conclusive in studies with those viruses that actually infect mice. For instance, it was shown that only complex gangliosides like GD1a, GD1b, GT1a, and GT1b (Fig. 2) mediate murine infection with mPyV, while shorter gangliosides from the same series bind the virus in in vitro studies (for instance, GD3). B4 KO mice, which lack functional GM2 synthase but produce the precursor of complex gangliosides GD3, are resistant to mPyV infection.<sup>5,29</sup> Similarly, GM2-deficient mice develop less dramatic hydrocephalus upon infection with reovirus T1L, which uses GM2 as a coreceptor.<sup>54,55</sup>

## 3. Identification of specific sialylated receptor candidates

### 3.1 Glycan arrays for verification of receptor glycan specificity

The specificities of viral capsid or attachment proteins for their glycan ligands are determined by factors such as glycan composition, shape and density of expression, and the involvement of nonglycan portions of the receptor, which may be part of the recognition motif. Many of these factors are difficult to determine experimentally. Glycan microarray, or glycan array, screening has become an essential tool in glycobiology, helping to unravel protein-carbohydrate interactions in many biological processes. In such arrays, multiple glycan structures are presented on a single slide to enable screening with glycan-binding proteins such as lectins, antibodies, bacteria, and viruses or virus capsid/attachment proteins. Bound proteins are then typically detected with fluorescently labeled antibodies. Different glycan arrays vary in glycan composition and in the way that the glycan structures are immobilized on the slides. Some arrays are based on covalent binding of amine-terminating glycans to N-hydroxysuccinimide (NHS)activated glass slides,<sup>56</sup> while others, so-called neoglycolipid (NGL)-based arrays, present glycans linked to lipids that are printed on nitrocellulosecoated glass slides.<sup>57</sup> The glycan microarray technology has been especially useful in defining the glycan receptor specificities of viruses.<sup>55</sup> The glycan receptors for a number of viruses were first identified using glycan array technology, including diverse sialylated compounds such as the linear lactoseries tetrasaccharide c (LSTc) structure for JCPyV,<sup>11</sup> the branched GD1a glycan for Ad37,<sup>16</sup> or the repetitive polysialic acid structure (polySia, Fig. 2) for Ad52.<sup>17</sup> Moreover, glycan array screening allows for fine differences in virus-glycan-binding preferences to be discerned, which is particularly useful when comparing the ligand-binding properties of closely related viruses or even subtly different strains of the same virus.<sup>58,59</sup> A particularly striking example for the ability of glycan microarrays to reveal differences in

recognition is provided by the analysis of the SV40 major capsid protein. Here, glycan arrays showed that GM1 terminating in Neu5Gc is a far better ligand for the virus than its Neu5Ac-based counterpart.<sup>60</sup> Humans and some other mammals have lost the ability to produce Neu5Gc from Neu5Ac, unlike Old World monkeys in which the respective hydroxylase enzyme gene, *CMAH*, is still intact (reviewed in ref. 61). Thus, this non-human virus specifically recognizes a sialic acid variant that is not synthesized by humans, which may, at least in part, determine its host range. Although SV40 was widely distributed as an unfortunate contamination of poliovirus vaccines in the 1950s and 1960s, no convincing association with human disease was established. Structural analyses of the SV40 capsid protein later confirmed that the protein surface has a specific recognition motif that can engage the additional hydroxyl group of Neu5Gc.<sup>62</sup> As expected, closely related human polyomaviruses specifically engage only Neu5Ac-based glycan receptor structures and lack this recognition motif.<sup>11,53</sup>

An interesting alternative to glycan microarray screening, at least for the comparison of binding profiles of related viruses or virus strains, could be the use of gold particles that have been functionalized with different glycan structures.<sup>63</sup> Such particles can be mixed with viral capsid proteins in solution, and binding can be evaluated using a colorimetric assay.

Despite the successful examples mentioned herein, it should be kept in mind that the technique merely aids identification of oligosaccharide structures that are able to engage a specific protein but does not allow evaluation of the physiological relevance of the observed interaction. Thus, other experimental approaches are always needed to validate hits obtained in glycan microarray screening.

#### 3.2 STD NMR spectroscopy for verification of receptor glycan specificity

Once a specific sialylated receptor candidate is identified by functional assays or in glycan microarrays, NMR spectroscopy can be employed to assess the exact binding epitope and subsequently delineate which other structurally related glycans could also serve as receptors. One NMR technique has been proven to be particularly useful in this regard, namely saturation transfer difference (STD) NMR spectroscopy. STD NMR experiments are comparably simple experiments as they require no isotope labeling and only relatively small amounts of virus or viral receptor-binding protein. We have, in the past, recorded NMR spectra for a number of members of the PyV and adenovirus families (i.e., their major capsid protein VP1) and sialylated receptor

candidates or structural proxies thereof and compared the resulting epitopes with crystal structures of the same complexes. In all but one case, results from both techniques agreed exceptionally well, yielding binding epitopes in solution for the SV40-GM1, BKPyV-GD3, MCPyV-GD3, TL1 σ1-GM2, JCPyV-GM1, TSPyV-GM1, and Ad52-polySia complexes.<sup>17,53,54,61,64,65</sup> Just like protein crystallography and microglycan arrays, NMR spectroscopy delivers atomic information about the minimal binding determinant of a given interaction, i.e., the minimal stretch of pyranoses, in the context of complex glycans. The reason for this is that "saturation transfer," the magnetic resonance phenomenon after which the technique is named, is strictly distance dependent. In the resulting NMR spectra the resonances (spectral peaks) from those sugar moieties that are in close proximity to the capsid or coat protein during the lifetime of the complex are, therefore, overrepresented.<sup>66</sup> For the PyVs named above, STD NMR spectra with the respective sialylated glycan receptor proxies (oligosaccharides) displayed particularly efficient saturation transfer to the protons of the sialic acid nonreducing end caps, or, in the case of the MCPyV-GD3 complex, for the second sialic acid residue from the nonreducing end (the  $\alpha$ 2-3 linked Neu5Ac rather than the  $\alpha$ 2-8 linked Neu5Ac ring<sup>64</sup>). Notably, even for polySia with its highly repetitive sequence ([Neu5Ac $\alpha$ 2-8]<sub>*n*</sub>), the reducing-end cap could be clearly identified as the major binding determinant of the Ad52 fiber knob protein because NMR chemical shifts are so sensitive to the proximate chemical environment that the reducingend Neu5Ac ring proton resonances in polySia can be unambiguously identified.<sup>17</sup>

Another virus family for which STD NMR spectroscopy has been applied successfully in this context are the rotaviruses.<sup>67–70</sup> For example, the sialidase-insensitive human Wa strain that was previously thought not to engage sialylated glycans was shown to bind sialic acid specifically on such glycans that are not completely cleavable by certain neuraminidases, such as the GD1a and GM1 ganglioside glycans (see Section 2.2).<sup>69</sup> Thus, the technique can also be used as a starting point in the identification of sialylated receptor candidates. While it is not practical to screen a large library of glycans for binding to virus capsids by STD NMR spectroscopy, the experiment can be helpful if an educated guess can be made as to which glycans may be relevant. For sialylated glycans, the simplest (and least expensive) representatives of the three most common Neu5Ac-linkage types are often sufficient, i.e., 3'sialyllactose (3'SL), 6'sialyllactose (6'SL), and the GD3 glycan (Fig. 2).<sup>71</sup>

#### 3.3 Affinity measurements

Due to the generally low-affinity regime, exact affinity values are difficult to determine experimentally using traditional biophysical approaches such as surface plasmon resonance or isothermal titration calorimetry. In some cases, virus–glycan affinities have been approximated using crystal soaking experiments.<sup>65,72,73</sup> Crystals of virus capsid proteins were soaked with sialylated glycans at different concentrations, and binding was quantified by integrating the experimentally observed electron density for sialic acid at these different concentrations. These analyses yielded affinities in the millimolar range for several virus–glycan systems, in line with rare examples where the affinities have been measured by more traditional means.<sup>62</sup>

#### 3.4 Differentiating between glycolipids and glycoproteins

In some cases, an oligosaccharide structure that has been shown to bind a virus is not lipid-linked but attached to a protein, usually via N- or O-linked glycosylation. Thus, the virus recognizes a glycoprotein with interactions that could include both glycan and protein residues. In the case of sialylated glycans, glycoprotein binding is likely when ganglioside supplementation assays do not restore cell attachment and infection of gangliosidedeficient cells. An example is the interaction of the Ad37 fiber with the GD1a glycan (Fig. 2), which was identified using glycan microarray screening.<sup>16</sup> Here, reduction of de novo ganglioside biosynthesis did not reduce virus binding, and GD1a ganglioside-containing liposomes did not compete with binding of virions to target cells. This suggested that the GD1a glycan must be linked to a protein, not a lipid. Removal of N-linked cell-surface glycans by treatment with N-glycosidase F (PNGase F) did not inhibit virus binding, indicating that the GD1a glycan is most likely attached via an O-linkage (and not an N-linkage) to a protein. This was corroborated through experiments that blocked de novo synthesis of O-linked glycans by pretreating the target cells with benzyl- $\alpha$ -D-GalNAc (see Section 2.3), which efficiently prevented virus binding and infection.

### 4. Atomic resolution structures of sialic acid-virus interactions and structure-based inhibitor design

#### 4.1 General aspects of virus-glycan structural biology

Technical developments such as single-photon pixel detectors for X-ray diffraction experiments and glycan microarrays for the identification of ligand candidates have accelerated the crystallographic analysis of virus- and viral protein-glycan interactions in the past decade. Structural and functional studies of virus-glycan interactions have also been facilitated by the increasing commercial availability of complex oligosaccharides. Viral coat and attachment proteins often display high symmetry-a considerable advantage when it comes to cocrystallization and crystal-soaking experiments with candidate glycans. Since glycan-binding sites are usually exposed and shallow, these regions in the target proteins often mediate crystal contacts, which are weak directed and undirected interactions between individual molecules within a protein crystal that allow for the formation of a relatively stable crystal lattice. However, the existence of several chemically equivalent glycan-binding sites in multivalent attachment proteins usually results in several crystallographically distinct binding sites in the context of the crystal lattice. While some binding sites may well be blocked by crystal contacts, others are usually available for glycan binding. There are also some cases where glycan conformation or orientation is clearly distorted by crystal contacts in which the glycan itself becomes involved. Thus, careful evaluation of crystal lattices, comparison of several binding sites within one crystal, possibly the evaluation of additional crystal forms, and complementation with additional biochemical techniques such as mutagenesis, glycan arrays, and STD NMR spectroscopy must be used to properly assess the physiological relevance of crystallographic models of viral protein-glycan complexes.

As can be seen from Table 1, many viruses are able to use a set of structurally related glycans rather than one specific glycan as the receptor-binding motif. Since it is difficult to represent the vast space of glycoconjugates present on mammalian cells with glycan microarrays, individual receptor profiles can be incomplete. A clever approach to help create a comprehensive picture of glycan receptor candidates for a given virus is computational carbohydrate grafting.<sup>74,75</sup> The approach compares the crystal structure or otherwise produced model of a protein-glycan complex to a virtual library of carbohydrates for which free energy-minimized conformations have been precalculated with the GLYCAM force field. This library is systematically searched for entries that contain the crystallographically observed glycan (sub)structure. Entries for which matching glycan epitopes are found can be subsequently scrutinized with respect to steric restrictions of the proteinbinding site, for example, by using docking algorithms or with molecular dynamics simulations. In this way, a list with potentially fitting glycans that are more complex than the crystallography-based "search model" is produced and can subsequently be scrutinized in additional crystallographic or other biochemical experiments. The computational carbohydrate grafting webtool can be assessed at http://glycam.org/djdev/grafting/.

#### 4.2 Single virus families

#### 4.2.1 Polyomaviruses

Polyomaviruses are small, nonenveloped double-stranded DNA viruses that can infect a large range of mammalian species and birds. Their viral genome typically encodes two regulatory proteins, the small and large T-antigens, and three structural proteins, VP1, VP2, and VP3. The major capsid protein VP1 forms pentameric capsomers, which assemble into a T = 7d icosahedral shell that encapsidates the viral DNA, as well as the minor capsid protein VP2 and, in some cases, also several copies of VP3 (Fig. 3). So far, more than 100 polyomavirus species have been found in samples taken from diverse mammals, birds, and fish<sup>76</sup> as well as invertebrates.<sup>77</sup> Of these viruses, 14 species have been proposed to be human-tropic, but only a subset has so far been directly associated with diseases, most often in immunocompromised individuals.

Polyomaviruses have served as models to understand the molecular events leading to cellular transformation and cancer since their discovery six decades ago. The human polyomaviruses JCPyV and BKPyV were discovered in 1971 and subsequently shown to be associated with progressive multifocal leukoencephalopathy (PML) and polyomavirus-associated nephropathy (PVN), respectively.<sup>78,79</sup> Their association with human cancers is a matter of vigorous debate.<sup>80–82</sup> Remarkably, 12 new human polyomaviruses have been identified in the last decade.<sup>83–93</sup> This explosion in the number of polyomaviruses associated with humans has arisen due largely to high-throughput genomic approaches to identify and sequence foreign DNA. Of these viruses, MCPyV is strongly associated with Merkel cell carcinoma (MCC), a rare and aggressive neuroendocrine cancer<sup>84</sup> that affects about 1500 persons per year in the United States.<sup>94</sup> Another new polyomavirus, TSPyV, was found associated with *Trichodysplasia* 



**Fig. 3** Schematic representations of the four virus particles discussed in this review. Shown are polyomavirus (A), reovirus (B), adenovirus (C), and coxsackievirus (D). The glycan-binding attachment protein of each virus is highlighted in *red*.

spinulosa.<sup>87</sup> This disease is seen almost exclusively in immunosuppressed transplant patients, and while it is not life threatening, it causes significant disfigurement. Human polyomavirus 9 (HPyV9) was identified in 2011 from the serum and urine of an asymptomatic kidney transplant patient<sup>88</sup> and a skin sample of a patient with MCC,<sup>89</sup> and its association with a human disease is unclear. St. Louis polyomavirus (STLPyV) and Malawi polyomavirus (MWPyV, HPyV10) were both isolated from stool samples and are closely related.<sup>90</sup> The human polyomavirus 12 (HPyV12) was described in a study focusing on the identification of new human polyomaviruses in the gastrointestinal tract, spleen, and lymph nodes.<sup>91</sup> The virus likely represents an early offshoot of a large and diverse clade comprising polyomaviruses from apes, bats, monkeys, rodents, and humans (MCPyV and TSPyV) in the initial phylogenetic analysis based on the polyomavirus large T-antigen (LT).<sup>91</sup> The New Jersey polyomavirus NJPyV was first discovered through the muscle biopsy in an organ transplant recipient with systemic vasculitis, myositis, and retinal blindness.<sup>93</sup> Histopathology analysis suggested a tropism for endothelial cells, and thus NJPyV may have contributed to muscle and ocular damage in the patient. Lyon IARC polyomavirus (LiPyV) was recently isolated from human skin swabs.<sup>92</sup> This virus is phylogenetically related to the raccoon polyomavirus PyV (RacPyV), which is associated with brain tumors in free-ranging raccoons. The remaining human polyomaviruses (HPyV6, HPyV7, WUPyV, and KIPyV) have not been associated with a specific disease, but they are similar to other polyomaviruses in that they are very prevalent in human populations as determined by serology.

Since they are required for the attachment and/or cell entry steps of many representatives of the polyomavirus family, sialic acid-containing glycans feature prominently in polyomavirus research. While sialylated gly-cans function as the only receptor for some family members, others require additional glycan or protein receptors for entry, and this has consequences for the entry process. The cell-surface receptors for the primate polyomavirus SV40, mPyV, and human BKPyV are all gangliosides—complex, sialic acid-containing sphingolipids that reside primarily in lipid rafts. SV40 uses GM1, whereas mPyV attaches to GD1a and GT1b, and BKPyV binds GD1b and GT1b (Fig. 2).<sup>2,8</sup> Both Neu5Ac- and Neu5Gc-containing GM1 variants can serve as receptors for SV40, but the presence of the additional hydroxy group in Neu5Gc significantly increases the affinity of the interaction.<sup>60</sup> The human polyomavirus JCPyV requires a serotonin receptor as well as the sialylated linear pentasaccharide LSTc for entry.<sup>11,12</sup>

While the role of the serotonin receptor is unclear, the JCPyV VP1 protein specifically engages the LSTc glycan in a characteristic L-shaped conformation.<sup>11</sup> MCPyV VP1 pentamers bind the ganglioside GT1b in vitro.<sup>14</sup> Less complex gangliosides like GM3, however, are sufficient for transduction of cultured cells with MCPyV pseudovirions, suggesting that all gangliosides with Neu5Acα2-3-caps can act as receptors.<sup>86</sup> The sialic acid-binding site of MCPyV VP1 has been structurally characterized and supports this notion.<sup>64</sup> However, in contrast to other PyVs, MCPyV requires a nonsialylated glycan structure, heparan sulfate, for initial attachment.<sup>15</sup> The available data suggest a model in which glycosaminoglycans and sialylated glycans act sequentially to promote cell attachment and entry.<sup>15</sup> TSPyV also engages sialic acid-based receptors but via a binding site on the capsid that is shifted with respect to previously determined sialic acid-binding sites on other VP1 variants. Cell-based studies<sup>65</sup> demonstrate the relevance of sialic acid engagement for attachment and infection, suggesting that glycolipids rather than N- and O-linked glycoproteins are important for infection. HPyV9 is especially closely related to the simian B-lymphotropic polyomavirus (LPyV), and both viruses engage short, sialylated oligosaccharides.<sup>53,58</sup> Interestingly, small yet important differences in specificity were detected: while HPyV9 VP1 preferentially binds α2-3-linked sialyllactosamine compounds terminating in Neu5Gc over those terminating in Neu5Ac, LPyV does not exhibit such a preference. As Neu5Gc cannot be synthesized by humans, it appears puzzling why the human HPyV9 would have evolved to bind to Neu5Gc. One possibility is that the virus exploits the dietary uptake of Neu5Gc-bearing compounds to help target it to specific tissues and facilitate spread, similar to what has been proposed for human toxins that preferentially engage Neu5Gc,<sup>95</sup> although in the case of HPyV9 the route of spread and tissue tropism are not studied well enough to support such an evolutionary model. Little is known about the receptorbinding properties of many of the more recently discovered polyomaviruses (KIPyV, WUPyV, HPyV 6-7). Structural analyses and glycan array screening of the VP1 capsomers of these viruses suggest that none of them bind sialylated glycans.<sup>96,97</sup>

Although many PyVs use sialylated glycans as their initial attachment receptors, they do not all use the same entry pathway. While JCPyV enters via clathrin-mediated endocytosis, other family members require caveolin for cell entry (Table 1). Notably, SV40 can be endocytosed and subsequently transported to the ER independent of clathrin and caveolae, instead depending on cholesterol and tyrosine kinase signaling. This somewhat atypical entry pathway proceeds via direct physical deformation and invagination of the plasma membrane upon multivalent interaction with the SV40 receptor GM1.<sup>3,4</sup> In fact, it seems that SV40 can use alternative entry pathways, depending on what is available on the cell line under investigation.<sup>98</sup> Since other members of the genus *Polyomaviridae* are similar in size and also depend on gangliosides for entry, the clathrin- and caveolaedependent entry may be common within this family.

Structurally, polyomaviruses engage their sialylated glycan receptors in shallow surface pockets at the outermost edge of the capsid. This exposed and, therefore, often antigenic part of the capsid surface is characterized by the BC, DE, and HI loops of the intertwined VP1 monomers. Unlike the well-conserved VP1 core structure, these loops exhibit high sequence variability among polyomaviruses, accounting for their different receptor specificities. For example, small changes in the binding pocket of mPyV lead to recognition of distinct sugar motifs, which are a crucial determinant of virus spread and tumorigenicity.<sup>5</sup> To date, X-ray structures of seven different polyomavirus VP1 proteins in complex with sialylated glycans have been reported. The human JCPyV, BKPyV, MCPyV, and HPyV9 viruses, but also the two simian viruses LPyV and SV40 and the murine virus mPyV, engage their respective sialylated glycan receptor motifs in the same general area of VP1 (Fig. 4). This shallow binding site is located at the interface between two monomers, with contributions from the DE, HI, and BC1 loops and the BC linker of one monomer and ccw BC2 and EF loops from the neighboring VP1 chain. In contrast, the recently determined structure of TSPyV shows that this virus engages Neu5Ac in a unique, exposed binding site that lies about 18A away from the sialic acid-binding groove of all other thus far investigated polyomaviruses. This new binding site is formed by the BC2 loop of a single VP1 monomer with no contributions from neighboring VP1 chains.<sup>65</sup> It is maybe not surprising to see such variation since virus-glycan interactions are usually of low affinity and depend on only a few specific contacts that can easily be modulated under evolutionary pressure.

In all structures of polyomavirus–receptor complexes, the bulk of the interactions with the capsid involve sialic acids, and thus polyomavirus–sialic acid contacts contribute most toward the affinity of the virus for its receptor. The remaining, nonsialic acid carbohydrates typically mediate only a few contacts to the protein, but these contacts can nevertheless be critical for specificity as they help cement interactions with a particular glycan and preclude interactions with others. Subtle VP1 amino acid changes in the



**Fig. 4** Sialic acid-binding sites in polyomaviruses. (A) Structure of a representative VP1 pentamer (PDB ID  $4U60^{99}$ ), with the binding sites for sialic acid highlighted in *magenta*. The ligand is shown in *ball-and-stick representation*. (B) Detailed view of the interactions of the terminal sialic acid bound to one of the binding sites shown in (A), belonging to TS polyomavirus. (C) Detailed view of interactions of an  $\alpha$ 2-8-linked di-sialic acid ligand bound to the VP1 protein of BK polyomavirus, another member of the polyomavirus family (PDB ID  $4MJ0^{53}$ ).

binding pocket can thus have a critical impact on infection and viral pathogenicity. This is illustrated by a designed single amino residue mutation in the binding pocket of human BKPyV, which results in a switch in the ganglioside receptor specificity from GT1b to GM1.<sup>53</sup> Affinity is also critical, as the closely related JCPyV binds several ganglioside motifs, including GM1 and GD1b, but the increased affinity for the  $\alpha$ 2–6-linked LSTc glycan is crucial for its function as a receptor.<sup>11,99</sup> Small differences in glycan specificity and affinity can, therefore, contribute in an essential manner to the cellular uptake and pathogenicity of polyomaviruses.

Due to their protruding, solvent-exposed structures, the loops that engage sialic acid receptors in polyomaviruses define much of the surface of the virus and are therefore also a natural target of the host's antibody response. Mutations in loop sequences can facilitate escape from immune surveillance while also altering the receptor-binding properties of the viruses. Such an antigenic drift is exemplified by JCPyV, a polyomavirus that is often found to be mutated in immunocompromised individuals. JCPyV establishes a persistent infection in the kidney of healthy individuals with no significant complications, suggesting that the virus is under immune surveillance. However, a number of studies have reported that viral isolates from PML patients contain mutations in the viral capsid VP1 protein. Many of the residues undergoing such mutations happen to be located in the sialic acid-binding site, and the mutant viruses have reduced affinity for the sialylated glycan receptor.<sup>100</sup> An attractive hypothesis therefore is that these PML-associated mutations might render the virus more pathogenic in infected hosts by allowing it to spread more readily to the brain due to reduced nonspecific attachment to sialic acid pseudo-receptors. The mutations might furthermore help the virus gain access to the central nervous system (CNS) by engaging an alternate, brain-specific receptor and then causing PML.<sup>101</sup>

#### 4.2.2 Reoviruses

Mammalian orthoreoviruses (reoviruses) are members of the Reoviridae family. Reoviruses contain 10 double-stranded (ds) RNA gene segments, which are enclosed in two concentric protein shells termed the outer capsid and the core (Fig. 3).<sup>102</sup> Reoviruses can infect the gastrointestinal and respiratory tracts of a variety of mammals but rarely cause systemic disease outside of the immediate newborn period. However, mounting reports of reovirus infections of humans in Asia<sup>103–105</sup> raise the possibility of emergence of more virulent strains. Most children are seropositive for human reovirus by the age of 5.<sup>106</sup> Reoviruses preferentially infect and lyse tumor cells and are being tested in clinical trials as oncolytic reagents for the treatment of a variety of cancers.<sup>107-113</sup> It is not yet clear why reoviruses infect tumor cells more efficiently than untransformed cells, but it is likely that distribution, accessibility, and density of cellular receptors contribute to this phenomenon. Enhanced understanding of reovirus interactions with receptors, especially glycans that are differentially expressed by many tumors, may lead to improved targeting of oncolytic viruses and reovirus-based vaccines. Reoviruses also serve as a versatile experimental system for studies of viral replication events at the virus-cell interface, including engagement of cell-surface receptors, internalization and disassembly, and activation of the innate immune response. They moreover provide a model system for studies of virus-induced apoptosis and organ-specific disease in vivo.

Reoviruses are about 80 nm in diameter and are composed of eight structural proteins. Five of these ( $\lambda 1$ ,  $\lambda 2$ ,  $\lambda 3$ ,  $\sigma 2$ , and  $\mu 2$ ) form the core, the crystal structure of which has been determined.<sup>114</sup> A second layer of proteins ( $\sigma 1$ ,  $\mu 1$ , and  $\sigma 3$ ) forms the reovirus outer capsid, with  $\mu 1$  and  $\sigma 3$  comprising the bulk of this capsid, and  $\sigma 1$  protruding from the

12 vertices of the icosahedron. The  $\sigma$ 3 protein serves as a protective cap for  $\mu$ 1, and cleavage of  $\sigma$ 3 by endosomal proteases during viral entry results in loss of  $\sigma$ 3 and generation of infectious subvirion particles.<sup>33</sup> The reovirus  $\sigma$ 1 protein serves as the viral-attachment protein.<sup>115,116</sup> Its tail partially inserts into the virion via "turrets" formed by the pentameric  $\lambda$ 2 protein, whereas the  $\sigma$ 1 head projects away from the virion surface, <sup>102,117</sup> where it can engage cell-surface receptors. There are high-resolution structures of overlapping domains of  $\sigma$ 1, <sup>118–120</sup> which has allowed an atomic-level model to be constructed of the entire protein.

The  $\sigma 1$  protein is partitioned into three functionally and structurally distinct domains: the tail, body, and head (Fig. 5). The N-terminal tail spans about 170 residues and forms an uninterrupted  $\alpha$ -helical coiled coil.<sup>120</sup>



**Fig. 5** Sialic acid-binding sites in reoviruses. (A) Structure of a portion of the  $\sigma$ 1 protein from T3D reovirus bound to  $\alpha$ 2-3-sialyl lactose (PDB ID 3S6X<sup>121</sup>), with the binding sites for sialic acid highlighted in *blue*. The zoom view (*lower left*) provides a detailed view of the bound sialic acid (*yellow*) and its interactions with the protein. (B) Structure of a portion of the  $\sigma$ 1 protein from T1L reovirus bound to the glycan moiety of ganglioside GM2 (PDB ID 4GU3<sup>54</sup>), with the binding sites for sialic acid highlighted in *pink*. The zoom view (*upper right*) provides a detailed view of the bound sialic acid (*yellow*) and its interactions with the protein. The *brown surfaces* in both proteins indicate the binding regions for the protein receptor JAM-A, which serves as a receptor for both T1L and T3D reoviruses.

The body domain comprises approximately 100 residues and primarily consists of  $\beta$ -spiral repeats.<sup>54,119,120</sup> The C-terminal 150 residues fold into the compact head domain composed of eight antiparallel  $\beta$ -strands that assemble into a jelly roll.<sup>118</sup>

Three major human reovirus serotypes have been described, which are represented by the prototype strains type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D). Animal studies have demonstrated that these strains differ dramatically in the route through which the viruses spread in infected hosts and in their pathogenesis. After infection of newborn mice, reoviruses disseminate to the CNS and produce serotype-specific patterns of disease.<sup>122</sup> Type 1 reovirus strains spread by hematogenous routes to the CNS where they infect ependymal cells, leading to nonlethal hydrocephalus.<sup>116,122,123</sup> In contrast, type 3 reoviruses spread primarily by neural routes to the CNS and infect neurons, causing fatal encephalitis.<sup>116,122–124</sup> The spread of type 2 reoviruses is not well understood, as this virus is difficult to propagate. The  $\sigma$ 1 protein plays a pivotal role in these distinct disease patterns,<sup>116,125</sup> most likely through the selective recognition of cell-surface receptors.

Reovirus binds to both proteinaceous and sialylated glycan receptors. All reovirus serotypes engage junctional adhesion molecule-A (JAM-A), a component of intercellular tight junctions, with high affinity.<sup>19,118,126,127</sup> JAM-A exists as a homodimer at the cell surface, and binding to  $\sigma$ 1 requires a monomeric version of JAM-A, as  $\sigma$ 1 engages the same surface that is used for JAM-A homodimerization. Reovirus also binds to cell-surface glycans bearing terminal sialic acid, and it is thought that these glycans function as coreceptors that establish initial contact and influence tissue tropism. Following attachment to JAM-A and carbohydrate receptors, reovirus internalization is mediated by  $\beta$ 1 integrins,<sup>128</sup> most likely via clathrin-dependent endocytosis.<sup>121</sup>

While the JAM-A binding site is conserved among all reoviruses, T1L and T3D reoviruses display distinct carbohydrate specificities and engage their glycan coreceptors in strikingly different locations. T3D  $\sigma$ 1 interacts with  $\alpha$ -linked Neu5Ac, and crystal structures of T3D  $\sigma$ 1 in complex with sialyllactose-based compounds terminating in  $\alpha$ 2-3-,  $\alpha$ 2-6-, and  $\alpha$ 2-8-linked Neu5Ac show that the glycan-binding site is in a region of the body domain, near the midpoint of the fiber (Fig. 5).<sup>119</sup> The N-terminal portion of the T3D  $\sigma$ 1 body engages Neu5Ac via a complex network of interactions that are identical for the three glycosidic linkages tested. Contacts include a bidentate salt bridge, which connects the arginine 202 side chain with the Neu5Ac carboxylate, and several augmenting hydrogen bonds and nonpolar

interactions. The additional sugar rings of the lactose backbone make minimal contacts with T3D  $\sigma$ 1, suggesting that T3D  $\sigma$ 1 likely recognizes a different sialylated carbohydrate sequence on the cell surface.

Reovirus displays serotype-dependent hemagglutination profiles. Type 1 reoviruses agglutinate human but not bovine erythrocytes, whereas type 3 reoviruses preferentially agglutinate bovine erythrocytes and agglutinate human erythrocytes less efficiently.<sup>34</sup> These observations suggested that the glycan-binding specificities of type 1 and type 3 reovirus are distinct, and this hypothesis was confirmed through the structural and functional analysis of T1L reovirus  $\sigma$ 1 in complex with its receptor, the GM2 ganglioside glycan.<sup>54</sup> GM2 binds in a shallow groove in the globular head domain of T1L  $\sigma$ 1 (Fig. 5). Both terminal sugar moieties of the GM2 glycan, Neu5Ac and N-acetylgalactosamine (GalNAc), form contacts with the protein, providing an explanation for the observed specificity for GM2. Most of the contacts are contributed by Neu5Ac, which is wedged into a cleft between  $\beta$ -strands B and C at the side of the  $\sigma$ 1 head, while the GalNAc docks onto a shallow protein surface using van der Waals interactions. Interactions between T1L  $\sigma$ 1 and GM2 are primarily comprised of hydrogen bonds between the sugar molecule and backbone atoms of the protein. A comparison of the T1L and T3D  $\sigma$ 1 crystal structures sheds light on the species-dependent differences in hemagglutination capacity. Whereas human erythrocytes express the Neu5Ac form of sialic acid,<sup>129</sup> bovine cells express mostly Neu5Gc and less Neu5Ac.<sup>130</sup> The additional hydroxyl group of Neu5Gc would face a hydrophobic pocket in the T1L  $\sigma$ 1 glycan-binding site, making a favorable interaction unlikely. In contrast, the T3D  $\sigma$ 1 binding site likely could accommodate either Neu5Ac or Neu5Gc.

The different locations of the carbohydrate-binding sites contrast with the conserved interactions of both  $\sigma 1$  proteins with JAM-A. The JAM-A-binding sites of both T1L and T3D  $\sigma 1$  proteins are located at the base of the head domain, and interactions between  $\sigma 1$  and JAM-A are similar in both serotypes.<sup>127,131</sup> If both protein- and carbohydrate-binding sites are accessible for both serotype 1 and serotype 3 reoviruses, it is possible that the attachment mechanisms are not conserved between the reovirus serotypes, which may contribute to the observed differences in viral tropism and spread.

#### 4.2.3 Adenoviruses

Adenoviruses are large, nonenveloped icosahedral viruses encapsulating a 35-kb double-stranded DNA genome. The 150-MDa particle has a

diameter of about 90 nm and is composed of three major capsid proteins: the hexon, the penton base, and the fiber (Fig. 3). Structural analyses of complete adenovirus particles have provided highly detailed insights into the organization of these proteins on the viral surface, and they have also revealed the roles of several minor capsid proteins in capsid stabilization.<sup>132,133</sup> The hexon protein is the major capsomer, forming 240 trimers. The pentameric penton base mediates virus internalization and is located at each of the 12 vertices of the capsid (Fig. 3).<sup>134,135</sup> The homotrimeric fiber serves as the viral-attachment protein. It protrudes from the penton base and consists of a globular head (the so-called knob), an elongated, fibrous shaft, and a tail. The knob is formed by three 8-stranded antiparallel β-sandwich domains that interact closely with each other.<sup>136</sup> The shaft features a repetitive structure, formed by concatenated  $\beta$ -spiral repeats, and varies significantly in length between the different adenovirus serotypes.<sup>18,137</sup> To date, more than 80 human adenovirus types have been identified and grouped into a total of seven species (A-G) according to their genome sequence and a number of functional criteria: serology, hemagglutination behavior, as well as their oncogenicity in rodents and their capability to transform primary human cells.<sup>138–140</sup> They can use a large variety of cell-attachment receptors and differ markedly in tropism and disease.<sup>141</sup> Due to their broad tropism and efficient gene-delivery properties, adenoviruses are promising gene-transfer vehicles in functional genomics and gene-delivery research.

Adenoviruses belonging to species A, C, D, E, F, and G attach to cells by engaging the coxsackievirus and adenovirus receptor (CAR),<sup>142–144</sup> while group B viruses either use the ubiquitously expressed membrane cofactor protein CD46 (MCP)<sup>145–147</sup> or the membrane glycoprotein desmoglein-2 (DSG-2) for attachment.<sup>148</sup> Members of several adenovirus species have more recently been shown to require sialylated glycans as a receptor. Some members of group D adenoviruses adhere to cells by engaging the GD1a glycan, which is likely attached to an unknown protein.<sup>16</sup> In addition, the group G adenovirus Ad52 engages polymeric chains of  $\alpha$ 2–8–linked sialic acid (polysialic acid).<sup>17</sup> Several additional receptors have been implicated in attachment of different adenovirus types, but little is known about the molecular details of these interactions.<sup>141</sup> The contact of the fiber with the primary receptor is followed with the engagement of integrin receptors by the adenovirus penton base protein, which then triggers virus uptake.<sup>149,150</sup>

The highly contagious disease, epidemic keratoconjunctivitis (EKC), is caused mainly by three group D adenoviruses (Ad8, Ad19, and Ad37) and is

recognized as a severe ocular disease for which no antiviral drugs are available. Common symptoms of EKC are pain, edema, lacrimation, and decreased vision that can last for months or even years. While it has long been established that Ad37 fiber knobs bind receptors terminating in sialic acid,<sup>51,73</sup> the precise nature of the sialylated glycan has remained elusive. Glycan array screening has recently revealed that Ad37 fiber knobs specifically recognize the oligosaccharide GD1a, a disialylated compound that features two branches, each terminating in sialic acid.<sup>16</sup> Structural analysis of the trimeric Ad37 fiber knob in complex with GD1a established that the two terminal sialic acid residues in GD1a bind to two different Ad37 fiber knob protomers in an identical manner, thus engaging two of the three possible binding sites simultaneously.<sup>16</sup> This bivalent interaction results in a 250-fold higher affinity compared to the monovalent sialyllactose-Ad37 knob interaction.<sup>73</sup> Thus, although each protomer in an Ad37 fiber knob would be able to bind sialic acid attached to different oligosaccharide structures, specificity for GD1a is generated by a bivalent interaction in which two protomers interact with the same receptor molecule (Fig. 6).



**Fig. 6** Sialic acid-binding sites in human adenoviruses. (A) Structure of a representative adenovirus fiber knob (adenovirus type 37, PDB ID  $3NOI^{16}$ ), with regions that can engage sialic acid in different knobs highlighted. The view is along the threefold axis. (B) Detailed view of interactions in the region colored *cyan* in (A), with the sialic acid bound to the Ad37 knob shown in *orange*. (C) Detailed view of interactions in the region colored *pink* in (A), with an  $\alpha$ 2-8-linked di-sialic acid ligand bound to the Ad52 knob (PDB ID 6G47<sup>17</sup>) shown in *orange*.

A different strategy to engage sialic acid-bearing glycan receptors is illustrated by the group G human adenovirus Ad52 (Fig. 6). This virus was isolated in 2003 from a small outbreak of gastroenteritis.<sup>151</sup> The virus diverged from other human adenoviruses and was classified into the new group Human mastadenovirus G, which otherwise exclusively contains Old World monkey adenoviruses. The adenovirus particle is normally equipped with only one fiber protein, but AdV-52, along with group F viruses Ad40 and Ad41, differs from all other known adenoviruses by the presence of two different fiber proteins, one short (coded by gene fiber-1) and one long (fiber-2).<sup>151-153</sup> The knob domain of the long fiber of Ad52 binds to CAR, while the knob domain of the short fiber was shown to bind sialic acid-containing glycoproteins on target cells.<sup>47</sup> The specific structures of the cellular sialic acid-containing glycans were only recently identified.<sup>17</sup> The short fiber knob of Ad52 specifically recognizes long chains of  $\alpha$ 2-8-linked sialic acids (polySia), an unusual posttranslational modification of selected carrier proteins. The virus can use polySia as a receptor on target cells. Structural analyses and structure-guided mutagenesis of the knob revealed that the nonreducing, terminal sialic acid of polySia engages the protein with directed contacts, and that specificity for polySia is further achieved through subtle, transient electrostatic interactions with additional sialic acid residues. Ad52 is the first example of a human virus for which polySia was shown to function as a cellular receptor. The role of nondirected electrostatic interactions observed for the Ad52 knob-polySia interaction provides a new aspect of virus-glycan interactions, and similar mechanisms may also underlie the attachment of other glycan-binding pathogens.

PolySia can be found in high expression levels on many tumors, and its expression is associated with high tumor aggressiveness and invasiveness, resulting in poor clinical prognosis.<sup>154–156</sup> Cancers expressing polySia are also often recurrent and nonresponsive to conventional treatments,<sup>154</sup> and, therefore, attention has been drawn to novel therapeutic approaches, including adenoviral vectors for gene delivery and the use of modified oncolytic adenoviruses. In a recent approach, the fiber knob of HAdV-5 was substituted with endosialidaseNF, a tail spike protein from the bacteriophage K1F to generate an efficient polySia-targeting oncolytic vector.<sup>157</sup> Although the low affinity of the interaction suggests a requirement for targeted mutagenesis for enhanced polySia binding, Ad52 could form the basis for a viable alternative strategy for developing oncolytic vectors, especially in light of the low seroprevalence rates and reduced liver tropism of this virus.<sup>47,158</sup>

#### 4.2.4 Picornaviruses

The Picornaviridae family is comprised of small, icosahedral, nonenveloped viruses with a single positive-strand RNA genome. Its members are among the smallest known human and animal pathogens. Picornaviruses relevant for human health include rhinoviruses, enteroviruses, hepatoviruses, coxsackieviruses, foot-and-mouth disease viruses, and polioviruses. The picornavirus capsid measures about 30nm in diameter and is composed of 60 copies each of four viral proteins VP1-4, which form a quasi T = 3 icosahedral shell (Fig. 7). The structures of several picornaviruses have been determined to near-atomic resolution. All show a similar structural pattern in which VP1–3 adopt an eight-stranded,  $\beta$ -barrel-type structure that forms the matrix of the shell. While VP1 lies close to a fivefold axis, VP2 is close to a twofold axis, and VP3 is close to a threefold axis of the capsid. VP4 lies inside the capsid and carries a myristyl group at its N-terminus. Most members of the family engage protein receptors such as decay-accelerating factor (CD55, DAF),<sup>30,160</sup> intercellular adhesion molecule 1 (ICAM-1),<sup>161</sup> the low-density lipoprotein receptor (LDL-R),<sup>162</sup> the CAR,<sup>163</sup> and integrins.<sup>164,165</sup> The DAF, CAR, and ICAM-1 protein receptors engage viral capsid residues that are located in a recessed "canyon" on the



**Fig. 7** Sialic acid-binding site in human coxsackievirus A24v. (A) Structure of pentameric VP1 core protein in intact coxsackievirus A24v particles (PDB ID 4Q4X<sup>159</sup>), with regions that can engage sialic acid highlighted in *pink*. Residues 25–72, 212–231, and 274–293 have been truncated for clarity. (B) Detailed view of interactions in one binding region, with the sialic acid bound to the capsid shown in *orange*. Residues labeled "cw" are contributed by the clockwise neighboring VP1 protein in the pentamer (when viewed from the outside) and are shown in *dark gray*.

virus surface.<sup>166</sup> Since the narrow canyon is inaccessible to most antibodies, it has been suggested that the recessed binding sites allow the virus to mutate exposed surface residues, thereby evading the host immune response, while keeping residues at the bottom of the canyon constant, thereby maintaining interactions with receptors.<sup>166</sup> A hydrophobic pocket underneath the canyon is the site of binding of various hydrophobic compounds that can inhibit attachment and uncoating. This pocket is also associated with an unidentified "pocket factor," possibly cellular in origin, but with unknown chemical identity. The pocket factor-binding site lies near the receptor-binding site, and the pocket factor can modulate receptor interaction. It is thought that interplay between the pocket factor and receptor molecules regulates the conformational changes required for the initiation of the entry of the viral genome into the cell.<sup>167</sup> However, not all picornaviruses engage their receptors via the capsid canyons. The LDL receptor-binding site on human rhinovirus 2 (HRV2), for instance, is located near the fivefold symmetry axes and does not bind to the canyon, for which it would also be too large.<sup>162</sup>

Sialylated glycans are required for cell attachment of three human picornavirus family members: coxsackievirus A24 variant (CVA24v), enterovirus 68 (EV68), and enterovirus 70 (EV70).<sup>168–170</sup> While EV68 causes mild-tosevere respiratory illnesses, CVA24v and EV70 have ocular tropism and are the causative agents of acute hemorrhagic conjunctivitis (AHC), a highly contagious eye infection.<sup>168,171</sup> During the last decades, CVA24v has been responsible for several AHC outbreaks and two pandemics.<sup>172–178</sup> Besides hemorrhagic conjunctivitis, CVA24v and EV70 can also cause symptoms in the cornea, upper respiratory tract, and neurological impairments such as acute flaccid paralysis.<sup>25,171,174,175</sup> Despite the recurring appearance of AHC, neither vaccines nor antiviral drugs are available for the prevention or the treatment of the disease.

The two AHC-causing viruses CVA24v and EV70 specifically engage glycans that terminate in Neu5Ac, with subtle differences in specificity. Cell binding and infection studies showed that EV70 binds Neu5Ac in the context of an  $\alpha$ 2–3 linkage,<sup>169</sup> CVA24v is able to use both  $\alpha$ 2–3– and  $\alpha$ 2–6–linked Neu5Ac as receptors, with some preference for the  $\alpha$ 2–6–linkage.<sup>168</sup> The sialic acid-containing receptor is used by CVA24v on corneal but not conjunctival cells.<sup>179</sup> A similar preference of  $\alpha$ 2–6–linked sialic acid glycans compared to  $\alpha$ 2–3–linked sialic acid compounds was very recently described in a glycan array analysis of EV68,<sup>170</sup> and this preference for  $\alpha$ 2–6–linked sialic acid might allow the virus to gain access to the upper respiratory tract, somewhat in analogy to influenza virus.

Recent high-resolution crystal structures of intact CVA24v complexed with a range of sialylated glycans provided the first view of a human picornavirus-sialic acid complex.<sup>159</sup> The Neu5Ac moiety binds near the fivefold axis, at a particularly solvent-exposed, protruding region of the virion, and it docks into a shallow, positively charged binding site formed by loops contributed by two monomers. Surprisingly, the sialic acid-binding site is distant from the recessed, canyon-like areas that engage protein receptors DAF,<sup>160,180</sup> CAR,<sup>162</sup> and ICAM-1<sup>160</sup> in other picornaviruses. Since the sialic acid-binding site in CVA24v does not include canyon residues and is in fact located at one of the most solvent-accessible regions of the virus, the site would be an easy target for a neutralizing antibody response. A survey of available virus-sialic acid complexes shows, however, that glycans typically bind to shallow, surface-exposed regions of the capsid proteins,<sup>96</sup> which is also the case for the examples presented in this work (Fig. 8). One can speculate about the underlying biochemical principles and evolutionary advantages of low-affinity glycan-binding sites that are surface-exposed and easy targets of the immune response. One possibility is that mutations that are enforced on the viral glycan-binding site by immune responses can, in the realm of protein-glycan interactions, be easily overcome without decreased fitness of the virus because single amino acid changes (which can dramatically impact immune recognition) can be coupled to receptor switches, i.e., targeting of another, structurally related receptor.<sup>9,53</sup> This phenomenon may be linked to the small number of directed contacts that underlie many glycan-recognition events in virology<sup>96</sup> and glycobiology in general.

Analysis of cocrystal structures of the CVA24v particle with a range of ligands shows that the preferred CVA24v receptor terminates in  $\alpha$ 2-6-linked Neu5Ac. However, the crystal structures alone do not offer a comprehensive explanation for the specificity for  $\alpha$ 2-6-linked Neu5Ac as good electron density was observed only for the Neu5Ac moiety in the CVA24v binding sites of all complexes, and both  $\alpha$ 2-6 and  $\alpha$ 2-3-linkages to additional carbohydrates (e.g.,  $\alpha$ 2-6-sialyllactose or  $\alpha$ 2-3-sialyllactose) could be accommodated. However, linear  $\alpha$ 2-3-linked sialyloligosaccharides such as  $\alpha$ 2-3-sialyllactose bound to a lesser degree to the virus, and they were also found to be less efficient in blocking virus binding than their  $\alpha$ 2-6-linked counterparts. One important difference between  $\alpha$ 2-6 and  $\alpha$ 2-3 linkages is, however, that  $\alpha$ 2-6-linked sialyloligosaccharides have increased flexibility compared to their  $\alpha$ 2-3-linked counterparts.<sup>182</sup> Molecular dynamics simulations of CVA24v demonstrated that the increased flexibility of  $\alpha$ 2-6-linked



**Fig. 8** Geometries and distances between sialic acid-binding sites in the multivalent capsid proteins of polyomavirus (A, VP1 pentamer), reovirus (B,  $\sigma$ 1 trimer), adenovirus (C, fiber knob trimer), and coxsackievirus (D, VP1 pentamer). In all cases, the distances are close enough to allow the design and synthesis of multivalent ligands that can potentially engage multiple sialic acid-binding sites. This has already been demonstrated for adenoviruss.<sup>181</sup>

glycans such as  $\alpha$ 2–6-sialyllactose yields a larger number of virus-to-receptor interactions, thereby likely favoring the binding of the virus to  $\alpha$ 2–6-linked glycans.

Thus, for the CVA24v–glycan interactions, as well as for the abovediscussed Ad52 knob–polySia interaction, high-resolution structures of the virus–glycan interaction were not sufficient to comprehensively understand receptor specificity. Both examples highlight how in the case of glycan receptors MD simulations can contribute significantly to our understanding of receptor specificity. This observation may be rooted in two striking differences between glycans and proteins as receptor molecules, namely (a) the generally much smaller size and therefore smaller binding interface of glycans and (b) the fact that for each glycan there are a number of closely related structural homologues in the same organism, which is not usually the case for protein receptors. The physiochemical effects that govern specificity in this very densely populated glycan chemical space may thus sometimes be subtle when compared to those exploited by the usually larger protein receptors, which cover larger interfaces and often have few close relatives.

#### 4.3 Structure-based inhibitors

Although they are fairly complex monosaccharides, sialic acids offer only limited opportunities for interactions. Hydrogen bonds can be formed to the hydroxyl and acetyl groups, and the carboxylate group can engage in salt bridges with positively charged amino acids. Often, however, the binding of carbohydrates merely results in the displacement of water molecules that form the same set of coordination and hydrogen bonds with the protein in unliganded structures, and thus the net energy gained by protein– carbohydrate hydrogen bonds is minimal. Hydrophobic interactions, which can contribute substantially to binding affinities, can only be made with the methyl group of Neu5Ac, and while this group is in fact often found to be inserted into small hydrophobic pockets in virus–receptor complexes,<sup>96</sup> it typically only forms smaller contact areas. All of these factors result in interactions between virus capsid proteins and sialic acid-based receptors that are typically of low affinity.

Targeting the sialic acid-binding site with a synthetic ligand that mimics contacts made by the sialic acid is therefore challenging. Nevertheless, some promising strategies for the development of antiviral compounds that are based on sialic acid have emerged. They exploit the observation that, in many cases, sialic acid-binding sites in individual subunits (viral capsid or attachment proteins) are located close to each other in the virus particle. This enables the design of multivalent ligands, bearing several terminal sialic acids that can engage with one binding site each, resulting in increased binding avidity. This strategy has recently been applied to develop inhibitors of the EKC-causing adenovirus Ad37. The trimeric fiber knob of this virus was shown to have three equivalent, proximate binding sites for sialic acid.<sup>73</sup> The crystal structure of the Ad37 fiber knob bound to the bi-antennary receptor glycan GD1a showed that the two terminal sialic acid residues, located on each of the two branches of the GD1a glycan, are accommodated into two out of three carbohydrate recognition sites on top of the Ad37 fiber knob.<sup>16</sup> The observed binding mode inspired the design of symmetric tri-sialic acid derivatives or glycoconjugates that can simultaneously bind to



**Fig. 9** Example of a multivalent (in this case trivalent) sialylated inhibitor bound to a viral capsid protein (in this case, the adenovirus 37 fiber knob).<sup>181</sup> The inhibitor molecule is simultaneously bound with its three terminal sialic acid residues. Also shown is a simulated annealing omit electron density map for the ligand (*grey* or *blue mesh*), contoured at 3  $\sigma$ . Panel (A) shows a view onto the trimer (surface rendering), and panel (B) shows a side view of the same trimer (ribbon drawing). The two views differ by 90 degrees.

all three fiber knob domains, thus improving the inhibitory potency in comparison to monovalent sialic acid compounds. The initially developed trivalent compounds were four orders of magnitude more potent than the natural sialic acid monosaccharide,<sup>181</sup> and more recently synthesized trivalent sialic acid inhibitors have been shown to be even more potent, inhibiting Ad37 adherence and infectivity at low nanomolar concentrations.<sup>183</sup> Analyses of crystal structures confirmed that the trivalent ligands do indeed bind as expected, with the three sialic acid moieties in a single compound engaging the three sialic acid-binding sites in a single fiber knob (Fig. 9).<sup>183</sup> The fiber knobs of EKC-causing adenoviruses, located at the most distal part of each of the 12 fibers that are protruding from the icosahedral virion, are highly homologous, and the critical sialic acid-interacting residues are conserved. Therefore, tri-sialic acid compounds offer promise as antiviral drugs for the topical treatment of EKC.

Since multivalency is a hallmark of sialic acid-binding viruses, the approach just described could also be adapted to other viruses by modifying the length of the linker and by changing the valency from tri- to penta-sialic acid in the cases of polyomaviruses and coxsackieviruses. Such a strategy would be especially attractive for inhibition of the coxsackievirus CVA24v, as this virus also causes an eye disease (AHC), and viral inhibitors could

therefore be administered with relative ease as topical treatment to prevent viral replication and spread. One should, however, keep in mind that targeting the sialic acid-binding sites of a sialic acid-binding virus with mimetics could trigger mutations in the capsid that might render the virus more pathogenic, as seen, for example, in the case of JCPyV.<sup>100,184</sup> Multitarget approaches may therefore be necessary to avoid fast development of drug resistance.

### 5. Outlook: The microbiome in enteric virus infections

In the last decade, the microbiome has emerged as a key factor in promoting enteric viral infection, with bacterial envelope glycosylation playing a decisive role. On a molecular level, quite different mechanisms seem to mediate the phenomenological observations that antibiotic treatment of mice and microbe-level filtering of stool samples used as infective reagents reduce infectivity of enteric human viruses like poliovirus and norovirus.<sup>185,186</sup> Direct bacterial effects like viral particle stabilization against environmental challenges by LPS (poliovirus, reovirus) as well as indirect effects through immune stimulation by bacteria (mouse mammary tumor virus) are reported.<sup>185,187,188</sup> Enrichment of viruses on bacterial envelopes was shown, suggesting that enteric viruses may piggyback through the microbiota-rich environment of the gut to reach their target cells in a way that not only facilitates passage but maybe also coinfection of one cell with several virus particles, potentially promoting genetic recombination.<sup>189</sup> Whatever the exact mechanisms, the phenomenon appears to depend heavily on bacterial glycosylation. The research field is relatively young and experimentally challenging, and thus the number of viruses for which solid data on the role of the microbiota exist is still small. Thus far, sialic acid has not been explicitly implicated in this particular aspect of glycovirology, but since there are sialylated commensals in the gut, and since the infectivity of the sialic acid-dependent enterovirus T3D reovirus in mice is reduced by antibiotic treatment,<sup>185</sup> it now seems merely a matter of time until an enteric virus is discovered for which sialic acid mediates a save passage rather than actual entry-or both.

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