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Sialic Acid Receptors of Viruses

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Abstract Sialic acid linked to glycoproteins and gangliosides is used by many viruses as a receptor for cell entry. These viruses include important human and animal pathogens, such as influenza, parainfluenza, mumps, corona, noro, rota, and DNA tumor viruses. Attachment to sialic acid is mediated by receptor binding proteins that are constituents of viral envelopes or exposed at the surface of non-enveloped viruses. Some of these viruses are also equipped with a neuraminidase or a sialyl-*O*-acetyl-esterase. These receptor-destroying enzymes promote virus release from infected cells and neutralize sialic acid-containing soluble proteins interfering with cell surface binding of the virus. Variations in the receptor specificity are important determinants for host range, tissue tropism, pathogenicity, and transmissibility of these viruses.

Keywords Ganglioside · Mucins · Neuraminidase · Receptor binding · Receptor-destroying enzyme · Sialate-O-acetylesterase · Virus

Contents

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1 Introduction

The initial step in the viral life cycle is the attachment of virus particles to the cell surface. Attachment is mediated by binding of the virus to a receptor. Sometimes co-receptors are also involved that might promote post-attachment events in the entry process. Receptor molecules are constituents of the cell membrane, and the receptor determinant, the structure to which the virus binds, may be either a protein epitope or the carbohydrate of a glycoprotein or a glycolipid. Soluble proteins present in body fluids and in mucus on respiratory and enteric epithelia may also contain such carbohydrates and therefore interfere with virus binding to the cell surface.

Sialic acid was the first virus receptor identified. Hirst and McClelland and Hare discovered that influenza virus is able to hemagglutinate and that adsorbed virus is eluted from erythrocytes on incubation at 37°C, indicating an enzymatic destruction of a receptor substance on the cells [1, 2]. When a similar enzymatic activity was subsequently detected in *Vibrio cholerae* cultures, the term "receptor-destroying enzyme" was introduced [3]. The substance released by the viral enzyme from soluble hemagglutination inhibitors was initially characterized as a carbohydrate of low molecular weight [4] and then identified in crystalline form as *N*-acetyl-D-neuraminic acid [5]. Thus, it was clear that the receptor determinant of influenza virus was sialic acid and that the viral enzyme was a neuraminidase. Furthermore, for the first time an important biological function of sialic acid had been identified.

Sialic acid has later also been found to serve as receptor of a large spectrum of other viruses. Most of them will be addressed here, with emphasis, however, on influenza viruses. For additional information we refer to several excellent reviews that have been published in recent years on similar topics [6–10].

2 Orthomyxoviridae

The orthomyxoviruses are enveloped viruses with a single-stranded, segmented RNA genome of negative polarity [11, 12]. There are five genera in the family: *Influenza virus A, B*, and *C*, *Thogotovirus*, and *Isavirus*. Influenza A viruses are further divided into subtypes characterized by 16 different hemagglutinins

(H1–H16) and 9 different neuraminidases (N1–N9). Except for the *Thogotovirus* genus, all orthomyxoviruses bind to sialic acid receptors. The receptor of an influenza A virus of subtype H17N10 isolated recently from bats [13] is not known.

2.1 Influenza A and B Viruses

Influenza A viruses are important human and animal pathogens. Their primary natural hosts are aquatic birds from which they are occasionally transmitted to other species. In man they cause outbreaks of respiratory disease that occur as annual epidemics and less frequent pandemics. Influenza B viruses are also believed to be descendants of avian influenza A viruses, but are now largely restricted to humans where they cause respiratory infections as well. Influenza A and B viruses have two envelope glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA), both of which interact with sialic acid.

2.1.1 Hemagglutinin

HA initiates infection by binding to cell surface receptors and by inducing fusion between viral and cellular membranes. HA is integrated in the virus envelope as a type I membrane protein. The ectodomain of HA represents 90% of the polypeptide chain. The residual 10% of the HA sequence accounts for the transmembrane domain and the cytosolic domain. HA is synthesized as a precursor molecule HA0 (75 kDa) which assembles to homotrimers. HA0 is *N*-glycosylated, palmitoylated, and proteolytically cleaved by host enzymes. The amino-terminal cleavage fragment HA1 (50 kDa) contains the receptor binding site and the carboxy-terminal fragment HA2 (25 kDa) is membrane anchored and responsible for fusion (reviewed in [14]).

The receptor determinant of influenza A and B viruses is sialic acid, mostly *N*-acetyl-neuraminic acid (Neu5Ac). The structures of complexes of HA of influenza A and B viruses with sialyloligosaccharides were determined by X-ray crystallography (reviewed in [15, 16]). The sialic acid-binding site is a shallow pocket located on the globular head of HA (Fig. 1). Virus binding depends not only on HA affinity for the terminal sialic acid residues, but also on the structure of the underlying oligosaccharide and protein or lipid moieties of the receptors, as well as on the abundance and accessibility of receptors on the cell surface. Because of this complex mode of binding, the receptor-binding properties of influenza viruses can be affected by amino acid substitutions inside the sialic acid-binding pocket, on the pocket rim, and by distant mutations resulting in altered glycosylation or altered electrostatic charge of the globular head of HA (reviewed in [17]). In natural glycoconjugates, sialic acids are α2-3- or α2-6-linked to Gal and GalNAc, α2-6-linked to GlcNAc, or α2-8-linked to the second Sia residue. Influenza viruses generally do not bind to α2-8-linked

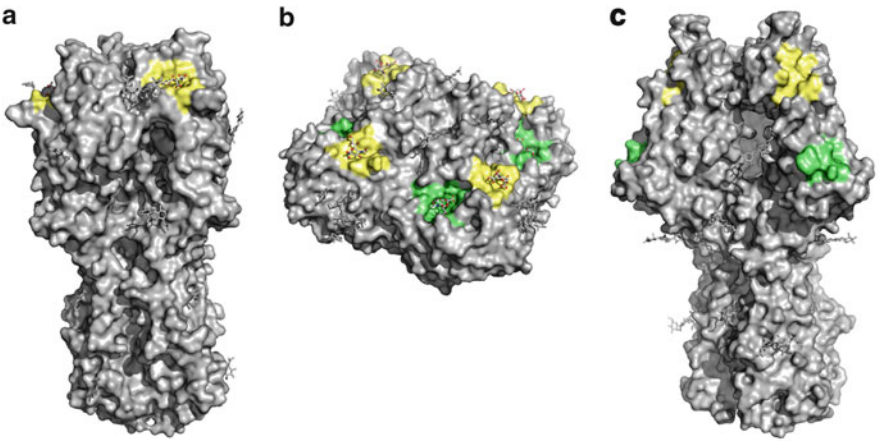

Fig. 1 Sialic acid binding sites of the hemagglutinin (**a**) and the neuraminidase (**b**) of influenza A virus and the hemagglutinin-esterase-fusion protein of influenza C virus (**c**). Molecular surfaces of HA and HEF trimers and the NA tetramer are shown. Receptor-binding sites of HA, HEF and the hemadsorption site of NA are colored *yellow*. The catalytic sites of NA and HEF are colored *green*. Sialic acid moieties in the binding sites of HA and NA are shown as stick models. The figure is based on crystal structures 1MQM, 1W20, and 1FLC from Protein Data Bank

Neu5Ac and can recognize only α2-3- or α2-6-linked sialic acid moieties such as Neu5Acα2-3/6Gal, Neu5Acα2-3/6GalNAc, and Neu5Acα2-6GlcNAc.

Differences in receptor-binding specificity of influenza viruses can contribute to viral host range restriction. Thus, human influenza viruses preferentially bind to α2-6-linked sialic acids (Neu5Acα2-6Gal), whereas avian influenza viruses preferentially recognize Neu5Acα2-3Gal [18–20]. These preferences are matched by predominant expression of Neu5Acα2-6Gal on epithelial cells in the human airway epithelium and by abundance of Neu5Acα2-3Gal on epithelial cells in the intestinal and respiratory tract of birds [21–26]. The receptor-binding specificity of human and avian influenza viruses suggests that avian viruses need to acquire the ability to recognize human-type receptors to be able to replicate efficiently and transmit in humans. Indeed, the earliest isolates of the 1918, 1957, and 1968 pandemics possessed HA that, although of avian origin, recognized human-type receptors (reviewed in [27, 28]). In light of these findings, the infection of humans with highly pathogenic avian H5N1 viruses seemed to be surprising as H5N1 viruses isolated from infected individuals preferentially recognize Neu5Acα2-3Gal [29–31]. Studies on human and avian virus infection in differentiated cultures of human airway epithelial cells indicated, however, that some cells in the human airway epithelium express sufficient amounts of receptors to allow infection with avian viruses and that receptor specificity determines the viral cell tropism in the epithelium. Early in infection, human viruses preferentially infected non-ciliated cells, whereas avian viruses mainly infected ciliated cells [32]. Other groups studied expression of viral receptors in human biopsies and archival tissues using lectins *Sambucus nigra* agglutinin, *Maackia amurensis* agglutinins I and II, and human and avian influenza viruses as molecular probes

[26, 33–36]. The results obtained in these studies suggest that paucity of receptors for avian viruses in the upper respiratory tract in humans is one of the factors preventing efficient human-to-human transmission. This concept is supported by recent studies showing that H5N1 mutants binding to α2-6 linked sialic acid are transmitted between ferrets through the air [37, 38].

Because pigs support replication of both avian and human viruses, they were considered to be a plausible intermediate host for the generation of human pandemic strains by gene reassortment (reviewed in [39]). This theory was further supported by the finding that both 3-linked and 6-linked sialic acid moieties were detected by staining on the histological sections of pig tracheal epithelium [23]. All early studies on swine influenza viruses were done using viruses that were grown in embryonated chicken eggs. However, similar to human influenza viruses, swine viruses appear to change their receptor specificity in eggs. Indeed, non-egg-adapted classical swine influenza viruses that were isolated and propagated solely in MDCK cells displayed a strict preference for 6-linked sialic acids and did not bind to 3-linked sialic acids [40]. This binding pattern is typical for non-egg-adapted human influenza viruses, and it is in discordance with the previously described ability of egg-adapted swine influenza viruses to recognize Neu5Acα2-3Gal [19, 23]. Thus, the receptor specificity of the pig viruses may be even closer to that of human viruses than originally thought. This notion agrees with recent data on a close similarity in the distribution of sialic acid receptors in the respiratory tract of pigs and humans [24, 26, 41].

The receptor specificity of the novel swine-origin H1N1/2009 pandemic influenza virus has been analyzed in studies employing carbohydrate microarrays. In some of these studies the virus was found to bind exclusively to α2-6-linked sialyl sequences [42–45], whereas in another study using a different microarray some binding to probes containing α2-3-linkages was also observed [46]. These studies showed also that the H1N1/2009 pandemic virus displayed the same binding profile as its putative swine precursors. The results indicate that no major change in receptor-binding specificity of HA was required for the emergent pandemic virus to acquire human-like characteristics and become established in the human population. Interestingly, mutations in the receptor-binding site of the HA of H1N1/2009 viruses have been detected sporadically, and the D222G substitution has been associated with severe or fatal disease [47, 48]. Compared to the parental virus, the D222G mutant virus displayed enhanced binding to α2-3-linked sugars [45, 49], infected a higher proportion of ciliated cells in cultures of human airway epithelium [49], and showed an altered pattern of attachment to human respiratory tissues in vitro, in particular increased binding to macrophages and type II pneumocytes in the alveoli [50]. These results suggested that the association of the D222G mutation with severe disease in humans reflects receptor-mediated alteration of the cell tropism of the mutant in human respiratory epithelium with enhanced replication in the lower respiratory tract.

Based on early data [18, 20, 51], it was assumed that all avian influenza viruses have similar receptor-binding specificity. The first evidence against this theory was obtained in a study showing that H5N1 viruses isolated in Hong Kong in 1997 from

poultry and humans had a lower receptor binding affinity and a lower neuraminidase activity than closely related viruses of aquatic birds [30].

Subsequent detailed receptor-binding studies revealed that influenza viruses adapted to ducks, gulls, and land-based gallinaceous poultry differ in their ability to recognize the sub-terminal saccharides of Neu5Acα2-3Gal-terminated receptors (reviewed in [28, 52]). In particular, duck viruses preferentially bind to receptors with type 1 and type 3 oligosaccharide sequences, such as Neu5Acα2-3Galβ1-3GlcNAc and Neu5Acα2-3Galβ1-3GalNAc, and viruses isolated from gulls show high-avidity binding to fucosylated sialyloligosaccharides Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc and Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc. In contrast, poultry-adapted viruses preferentially bind to receptors with type 2 sequences, such as Neu5Acα2-3Galβ1-4GlcNAc, with particularly strong binding to the corresponding sulfated analogues Neu5Acα2-3Galβ1-4(6-O-HSO_3)GlcNAc and Neu5Acα2-3Galβ1-4(Fucα1-3)(6-O-HSO_3)GlcNAc. Furthermore, some viruses of the Eurasian lineage of H9N2 poultry viruses bind to Neu5Acα2-6Gal terminated sialyloligosaccharides [28]. Thus it seems that influenza viruses circulating in different birds can have different receptor specificity owing to distinctions between the sialic acid receptors in these avian species.

2.1.2 Neuraminidase

NA is a type II membrane protein that is present in homotetrameric form in the viral envelope [53]. Each monomer consists of a cytoplasmic tail six amino acids in length, a stem region varying in length between 19 and 45 amino acids, and a carboxy-terminal globular head [53, 54]. The monomers are linked to dimers by disulfide bridges in the stalk region. The available evidence indicates that the neuraminidase has several functions in the life cycle of influenza virus. It was Burnet who proposed more than 60 years ago that the RDE allows the virus to penetrate the mucus layer coating the respiratory epithelium and thus to infect its target cells [3]. This concept has recently been shown to be correct when it was found that the neuraminidase inhibitor oseltamivir prevented initiation of infection of human tracheo-bronchial cell cultures [55]. The second function of the neuraminidase is at the end of the life cycle where it promotes virus release and prevents clumping of virions by removing receptors from the cell surface and viral glycoproteins, respectively [56].

Interspecies transmission of avian influenza viruses from aquatic birds to terrestrial poultry is often accompanied by a deletion in the stalk region of the NA and reduced catalytic activity [30, 57]. The observation that the reduced catalytic activity of NA is compensated by mutations in HA resulting in decreased receptor affinity led to the concept that optimal virus replication depends on a balance between receptor binding by HA and receptor destruction by NA [58–61].

The catalytic site of NA is located in the globular head region (Fig. 1). It is in the center of a propeller-like structure formed by four anti-parallel β-sheets [53]. *N*-Acetyl-neuraminic acid is bound by hydrogen bonds to amino acids

R118, D151, R152, R224, E276, R292, and R371 (N2 numbering). The acetamido group is linked by van der Waals forces to W178 and I222. The amino acids directly interacting with sialic acid are stabilized by contacts with amino acids E119, R156, S179, D/N198, N294, and E425. All of these amino acids are conserved among different NA subtypes.

NAs of avian influenza viruses have, in addition to the catalytic function, the capacity to agglutinate erythrocytes [62–64]. NAs of human viruses are unable to hemadsorb. The hemadsorption site is a shallow pocket located close by, but separately from to the deep catalytic site (Fig. 1) [65]. It is formed by three amino acid loops, with residues S367, S370, and S372 in the first, N/I400 and W403 in the second, and E/K/Q/N432 in the third loop, directly interacting with the sialic acid moiety. Recently it could be shown that the hemadsorption function enhances the catalytic activity of NA. This study also revealed that the hemadsorption activity of the NAs of early human isolates of the pandemics of 1918 and 1957 was reduced or completely absent. Thus, it appears that loss of the hemadsorption site is the result of an adaptive mutation involved in interspecies transmission from bird to man and has therefore to be considered as a pandemic marker [66].

2.2 *Influenza C virus*

Influenza C viruses that cause mild respiratory infections in humans differ from other influenza viruses because (1) their preferred sialic acid is *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2), (2) their receptor-destroying enzyme is an acetylesterase rather than a neuraminidase, and (3) three functions are combined in one surface glycoprotein, the hemagglutinin-esterase-fusion (HEF) protein: sialic acid binding, esterase and membrane fusion activity as compared to influenza A and B viruses where sialic acid binding and neuraminidase activity are distributed on two glycoproteins, the HA and NA proteins.

The HEF protein is a type I membrane protein of about 80 kDa [67, 68]. It is synthesized as a precursor (HEF0) that is post-translationally cleaved into the subunits HEF1 and HEF2. HEF1 comprises the sialic acid-binding and esterase activity and is connected via disulfide bonds to the membrane-bound HEF2 subunit. Despite little sequence similarity, HEF and HA show surprising structural similarity. The receptor domain of HEF is inserted into a surface loop of the esterase domain and the esterase domain is inserted into a surface loop of the stem which includes the hydrophobic peptide at the aminoterminus of HEF2 that is crucial for the fusion activity [69]. The sialic acid binding site is a cavity at the tip of each HEF1 subunit. The active site of the acetylesterase is located at the base of the globular head region. In the viral spikes HEF is present in homotrimeric form [69] (Fig. 1).

2.2.1 The Esterase Activity of HEF

The receptor-destroying enzyme of influenza C viruses was identified as an acetylesterase that releases the 9-*O*-acetyl residue from Neu5,9Ac$_2$ [70]. No or little activity was observed when the *O*-acetyl groups were linked to C-4 or C-7 of sialic acid. The enzyme belongs to the class of serine hydrolases with a catalytic triad formed by residues S57, D352, and H355 [69, 71–73]. The biological importance of the acetylesterase activity of HEF is believed to be similar to that of the neuraminidase of influenza A virus, i.e., facilitating virus spread by inactivation of potential receptor determinants from the surface of the infected cells and from the viral surface. In the initial stage of the infection cycle, the receptor-destroying enzyme may facilitate virus entry, e.g., by enabling virus to penetrate the mucus layer covering the respiratory epithelium [74]. In the late stage of the growth cycle, inactivation of receptor determinants may promote release of viruses from the infected cell and may prevent the formation of virus aggregates [75]. Supporting evidence has been provided by studies involving enzyme inhibitors, sialic acid analogues, and de- and resialylation experiments [73, 76, 77].

2.2.2 Sialic Acid Binding Activity of HEF

The identification of the receptor-destroying enzyme of influenza C virus as a sialate 9-*O*-acetylesterase indicated that Neu5,9Ac$_2$ is a receptor determinant for this virus [70]. Formal proof for the importance of 9-*O*-acetylated sialic acid was provided by desialylation and resialylation of cultured cells which abolished and regenerated agglutination of erythrocytes [78] as well as susceptibility of cultured cells to infection by influenza C virus [79]. The results demonstrated the role of Neu5,9Ac$_2$ for the cell tropism of the virus. As sialic acids are present on many cell surface glycoconjugates, attempts to identify a specific receptor for virus infection have failed so far for influenza A and B viruses. In the case of influenza C virus, overlay binding assays with immobilized membrane proteins indicated that the major interaction partner on the surface of the susceptible cell line MDCK I is gp40, a mucin-type glycoprotein with a high content of *O*-glycans [80, 81].

Crucial amino acids for substrate binding are residues Y127, T170, and G172 [69]. The specificity for the 9-*O*-acetyl group is determined by Y224 and R236 that interact with the carbonyl oxygen and by residues W225, W293, and P271 that form a pocket for the methyl group. Interestingly, influenza C virus can adapt to growth in cells with a low content of Neu5,9Ac$_2$. Passage in such cells or establishment of a persistent infection resulted in viruses with increased binding affinity to 9-*O*-acetylated sialic acids. These mutants or variant viruses had mutations at residues 269, 270, or 272, i.e., next to the above-mentioned P271 [82–85].

2.3 *Isavirus*

Infectious salmon anemia virus (ISAV) is an important pathogen in farmed Atlantic salmon. Similar to influenza viruses it has a hemagglutinating and a receptor-destroying activity. Unlike influenza A and B viruses, the RDE is not a neuraminidase but an acetylesterase [86]. The enzyme belongs to the class of serine hydrolases [86, 87]. Unlike the HEF protein of influenza C virus, the ISAV esterase releases the 4-*O*-acetyl group of 4-*O*-5-*N*-acetylneuraminic acid (Neu4,5Ac_2) [88]. This enzymatic activity corresponds to the preferred ligand of the ISAV hemagglutinin which is also Neu4,5Ac_2 [88]. Both the sialic acid binding activity and the acetylesterase activity are functions of the 38–43-kDa surface glycoprotein which has been designated HE protein [89–91].

3 Coronaviridae

Coronaviruses (order *Nidovirales*, family *Coronaviridae*) are a diverse group of viruses that cause enteric, respiratory, and neural infections in both mammalian and avian species. According to a current proposal to the International Committee of Taxonomy of Viruses, they are classified within the subfamily *Coronavirinae* which comprises four genera: *Alpha-, Beta-, Gamma-*, and *Deltacoronavirus*. The diversity of coronaviruses is also evident in the sialic acid binding activity. Some members of the *Betacoronavirus* genus, e.g., bovine coronavirus (BCoV), recognize *O*-acetylated sialic acids and contain an acetylesterase that functions as a receptor-destroying enzyme. On the other hand, some alpha- and gammacoronaviruses lack a comparable enzyme and have a preference for *N*-acetyl- or *N*-glycolylneuraminic acid, the best studied examples being transmissible gastroenteritis virus (TGEV) and infectious bronchitis virus (IBV). In addition to the above-mentioned viruses, both alpha- and gammacoronaviruses also include members that lack any sialic acid binding activity, e.g., SARS coronavirus and human coronavirus 229E. In the following, the sialic acid binding activities of BCoV, TGEV, and IBV will be described in more detail.

3.1 *Betacoronaviruses*

The presence of an acetylesterase in coronaviruses was first described by Vlasak and coworkers who showed that BCoV and HCoV-OC43 eluted from the erythrocytes during the course of a hemagglutination reaction, rendering the cells resistant to subsequent agglutination by either of the two coronaviruses or by influenza C virus. This finding demonstrated that BCoV and HCoV-OC43, similar to influenza C viruses, have a sialate 9-*O*-acetylesterase that functions as a receptor-destroying enzyme [92].

The acetylesterase activity was assigned to the HE surface glycoprotein of BCoV, hemagglutinating encephalomyelitis virus (HEF), and mouse-hepatitis virus [93–95]. The three-dimensional structure of the HE protein of BCoV has been determined showing an esterase site similar to that of the influenza C virus HEF protein [96]. By contrast, the sialic acid binding site of HE differs from that of the HEF protein with the ligand bound in the opposite orientation. An HE gene is present only in members of the *Betacoronavirus* genus. The different strains of murine coronaviruses contain an HE gene but differ widely in the amount of protein expressed. The acetylesterase of murine coronaviruses has been shown to have a different substrate specificity compared to that of BCoV, HEV, and HCoV-OC43, which release the *O*-acetyl residue from position C-9 of sialic acids. By contrast, murine coronaviruses – with the exception of the diarrhea virus of infant mice [97] – preferentially hydrolyze the ester linkage of 4-*O*-acetyl-*N*-acetylneuraminic acid [98–100].

The biological role of the acetylesterase of the betacoronaviruses is assumed to be similar to that of the receptor-destroying enzymes of influenza viruses, i.e., it may inactivate binding sites for the virus (1) on the cell surface and thus allow virus release from the infected cell, (2) on mucins covering the respiratory epithelial cells and thus facilitate the penetration of the mucus layer, and (3) on viral surface glycoproteins or glycolipids and thus prevent aggregate formation. Conflicting data have been reported concerning the role of the receptor-destroying enzyme in the initial stage of infection. Inhibition of the acetylesterase by diisopropyl fluorophosphate was shown to reduce the infectivity about a hundredfold in one report, and to have no effect in another report [94, 101].

Following the discovery of an acetylesterase in BCoV and HCoV-OC43 [92] it has been shown that 9-*O*-acetylated sialic acid serves as a receptor determinant not only for binding to erythrocytes but also for initiating infection of cultured cells [102]. When polarized epithelial cells such as MDCK I cells were analyzed for susceptibility to infection, BCoV was found to infect the cells via the apical but not via the basolateral side of the membrane [103, 104]. The inability of BCoV to infect MDCK I cells via the basolateral plasma membrane may reflect that the major glycoprotein recognized by BCoV, a mucin-like glycoprotein of 40 kDa, is predominantly present in the apical membrane domain [105]. An alternative explanation is that BCoV requires an additional receptor for initiation of infection, which is present only on the apical membrane. Such a secondary receptor has not yet been identified for BCoV.

The HE protein of BCoV has not only acetylesterase activity (see above); it can also function as a hemagglutinin [93, 106, 107]. However, BCoV agglutinates a wider spectrum of erythrocytes than does the isolated HE protein. HE only agglutinates cells that contain a high content of $Neu5,9Ac_2$ such as mouse and rat erythrocytes. Chicken erythrocytes are agglutinated by BCoV and HCoV-OC43, but not by the HE protein. The second surface glycoprotein of BCoV, the S protein, has an important function in virus entry by being involved in the attachment of virions to the cell surface and by mediating the subsequent fusion of the viral and the cell membrane. By contrast to the HE protein, isolated S protein is able to

agglutinate chicken erythrocytes [108]. Therefore, the S protein of these viruses is the major hemagglutinin and thus the major sialic acid binding protein. With murine coronaviruses, where the role of *O*-acetylated sialic acids as an essential receptor determinant has not been demonstrated [109], a sialic acid binding activity could be assigned only to the HE protein, not to the S protein [110].

3.2 Alphacoronaviruses

TGEV is an enteropathogenic virus which may affect pigs of all ages. Infections are especially severe in piglets up to two weeks of age which usually die unless they are protected by maternal antibodies. When Noda and co-workers [111, 112] first described the ability of TGEV to agglutinate erythrocytes, the virus appeared to contain a weak hemagglutinin. This is probably related to the absence of a receptor-destroying enzyme that may remove competitive inhibitors from the viral surface. In fact, when a virus or cells used for virus growth were pre-treated with neuraminidase, the resulting virions were able to agglutinate erythrocytes efficiently. In this way it was shown that the HA-activity of TGEV was due to a sialic acid-binding activity with a preference for α2-3-linked *N*-glycolylneuraminic acid [104, 113].

The sialic acid binding activity of TGEV is located in the amino-terminal portion of the surface glycoprotein S between amino acids 20 and 244. Evidence is based on the hemagglutination-inhibiting effect of monoclonal antibodies and on the analysis of mutant proteins with one or more amino acid exchanges [104, 113]. Interestingly, all mutants that had lost hemagglutinating activity were strongly reduced in their enteropathogenic effect, indicating that the sialic acid binding activity is an important factor for the enteropathogenicity of TGEV [113–115].

In virus overlay binding assays with brush border membranes from suckling piglets, TGEV recognized a high molecular mass protein via its sialic acid binding activity [116]. This highly glycosylated protein was designated MGP (mucin-like glycoprotein) as it possesses typical characteristics of a mucin. In in situ binding assays with jejunal cryosections, TGEV bound in a sialic acid-dependent manner to a component that was mainly localized in the goblet cells which are known to synthesize and secrete mucins [116]. From these data it can be concluded that binding to the sialic acids of MGP helps the virus to penetrate the mucus layer and to proceed to the intestinal enterocytes for initiation of infection.

This explanation also applies to an interesting phenomenon related to TGEV. A respiratory variant of TGEV, the porcine respiratory coronavirus (PRCoV), was first isolated in Belgium [117] and found to be very similar to TGEV. The major difference was a deletion of 224 amino acids in the N-terminal half of the S protein. Both TGEV and PRCoV use pAPN (porcine aminopeptidase N) as a receptor to infect their host cells [118]. In contrast to TGEV, the S protein of PRCoV displays no hemagglutinating activity as the sialic acid binding site is located in the deleted region of the S protein [104]. PRCoV does not replicate efficiently in the gut [119]. As the S proteins of TGEV and PRCoV share the binding sites for

neutralizing antibodies, the spread of PRCoV in European pigs acted like the spread of a vaccine virus, resulting in drastic reduction of TGEV infection. Though PRCoV, similar to TGEV, uses pAPN as a cell surface receptor for entering host cells, PRCoV, unlike TGEV, is not an enteropathogenic virus. As in the case of the mutants mentioned above, the lack of sialic acid binding activity appears to be responsible for the lack of enteropathogenicity.

Though sialic acids are the receptor determinants for the HA activity of TGEV and are crucial for the enteropathogenicity of the virus, the sialic acid binding activity appears to be dispensable for growth of the virus in cell culture. TGEV mutants deficient in sialic acid binding activity grow well in cell culture using pAPN as receptor [113, 115]. However, in binding assays the amount of parental virus attached to sialic acids on the cell surface was increased sixfold compared to mutant virus that was only able to bind to pAPN [120]. Recent results demonstrated that binding to sialic acids is dispensable for infection of cultured cells, when a conventional adsorption time is applied, i.e., 60 min. However, when the adsorption time is reduced to 5 min, infection becomes sialic acid-dependent, as indicated by the effect of pretreatment of cells with neuraminidase, which resulted in a more than 80% reduction of infectivity. This result indicates that the sialic acid binding activity can facilitate infection under unfavorable conditions [121] and therefore may be necessary for infection of the intestine.

3.3 Gammacoronaviruses

Bingham and coworkers [122] reported that some IBV strains were able to agglutinate erythrocytes. Similar to TGEV, IBV requires pretreatment with neuraminidase for efficient hemagglutinating activity. Furthermore, it preferentially recognizes α-2-3-linked sialic acid [123]. Recently, it has been shown that sialic acid is also a crucial receptor-determinant for infection of cells [124]. Pretreatment with neuraminidase was found to result in decreased infectivity as indicated by a reduced number of infected cells and by lower titers of virus released into the supernatant. This finding was obtained with both a lab strain and strains circulating in poultry [124–126]. The sialic acid-dependence of the IBV infection was observed both with conventional cell cultures and differentiated airway epithelial cells from trachea and lung [126, 127].

3.4 Torovirus

Toroviruses belong to the family *Coronaviridae* and are classified within the subfamily *Torovirinae* and the genus *Torovirus*. They cause mild infections of swine and cattle [6]. Toroviruses contain an HE protein that resembles the HE proteins of betacoronaviruses [128, 129]. Like the coronaviral counterparts,

torovirus HE proteins are acetylesterases. The enzyme of bovine torovirus releases the *O*-acetyl group from position C-9 of sialic acid and accepts as a substrate both Neu5,9Ac_2 and *N*-acetyl-7(8),9-*O*-acetylneuraminic acid; this specificity resembles those that have been reported for the HEF protein of influenza C virus and for the HE proteins of several coronaviruses [100]. By contrast, the HE protein of porcine torovirus has a narrower specificity, accepting Neu5,9Ac_2 but not Neu5,7(8),9Ac_3 as a substrate [100]. Analysis of the crystal structure revealed that the torovirus HE proteins have an esterase domain similar to those of the coronavirus HE and influenza C virus HEF proteins [130]; on the other hand, the sialic acid binding site is unique. The difference in substrate specificity is explained by a single amino acid, Thr73 in the porcine and Ser64 in the bovine HE protein [130].

4 Paramyxoviridae

The *Paramyxoviridae* family that is divided into two subfamilies and seven genera comprises a large group of enveloped viruses with non-segmented single-stranded RNA genomes of negative polarity. The members of the genera *Respirovirus, Rubulavirus,* and *Avulavirus* are viruses that share binding to sialic acid-containing cell receptors as a common feature. They include several major pathogens for man (human parainfluenza viruses (HPIV) 1-4, mumps virus) and animals (Newcastle disease virus (NDV)) as well as Sendai virus that became an important tool in genetic engineering because of its capacities as a membrane fusing agent and a gene vector.

Receptor interaction of these viruses is mediated by the hemagglutinin-neuraminidase (HN) glycoprotein, a type II integral membrane protein with an N-terminal cytoplasmic tail, a transmembrane domain, a membrane-proximal stalk domain, and a large C-terminal globular head domain that contains the sites responsible for hemagglutinating and neuraminidase activities. HN forms tetramers that are present as spikes on the surface of the virus particles (see [131]).

HN is believed not only to initiate infection by receptor binding but also to prevent aggregation and to promote release of mature virions by receptor removal. X-Ray crystallographic analysis of the HN glycoprotein of NDV [132], HPIV3 [133], and SV5 [134] has revealed a typical neuraminidase fold consisting of six antiparallel β strands organized as a super barrel with a centrally located active site located at the tip of the globular head domain. This exerts both the receptor binding and the catalytic function. A second sialic binding site has been observed on HN of NDV, the biological function of which, however, has not been clearly established yet [132]. HPIV1 HN also has a second binding site, but it is accessible only after removal of a nearby carbohydrate side chain [135].

The receptor specificity of Sendai virus was first analyzed in studies employing gangliosides [136, 137] and erythrocytes that contained defined sialyloligosaccharides after neuraminidase and subsequent sialyltransferase treatment [138]. These studies showed that Sendai virus has a preference for α2-3-bound

N-acetylneuraminic acid. This receptor determinant appears to be present on both glycoproteins and gangliosides [139, 140]. HPIV1 also recognizes α2-3 linkages, whereas HPIV3 has α2-6 specificity [141].

5 Caliciviridae

Caliciviruses are small non-enveloped viruses that contain a single-stranded plus-sense RNA genome encapsidated by an icosahedral protein shell. The major capsid protein VP1 has a shell (S) domain and a protruding (P) domain [142, 143]. The P domain which forms arch-like structures on the virion surface is further subdivided into subdomains P1 and P2. P2 is the most variable region and contains carbohydrate binding motifs [144–148].

Caliciviruses which occur in a large variety of different hosts are subdivided into several genera, including the genus *Norovirus*. Human noroviruses are responsible for the majority of acute viral gastroenteritis. Although these infections are usually mild they can be a serious threat to the elderly and the immuno-compromised. Murine noroviruses share pathogenic properties with human noroviruses as they are enteric viruses that replicate in the intestine and are shed in feces [149].

Whereas most human noroviruses bind to non-charged histo-blood group antigens [150–152] or to heparan sulfate [153], some recognize sialyl-Lewis X neoglycoproteins. Binding to the sialyl-Lewis X group is strictly sialic acid-dependent, since a non-sialylated control glycan does not bind [154]. While the tropism of human norovirus remains unknown, murine noroviruses efficiently replicate in murine macrophages and dentritic cells [149]. Virus binding to the macrophage surface is partially neuraminidase-sensitive and ganglioside-dependent [155]. Murine macrophages express gangliosides GD1a and GM1, and murine norovirus binds to GD1a, but not to GM1, suggesting that the minimal binding epitope is the terminal sialic acid found in GD1a [148]. Only in a few other instances has sialic acid been identified as a calicivirus receptor. Thus, a feline calicivirus strain attaches to α2-6-linked sialic acid on *N*-glycans [156].

6 Picornaviridae

Among the *Picornaviridae*, a large family of non-segmented positive-stranded RNA viruses comprising many animal and human pathogens, the use of sialic acid as a receptor component has been described for encephalomyocarditis virus [157], Theiler's murine encephalomyelitis virus (TMEV) [158], mengovirus [159], and bovine enterovirus 261 [160]. A human enterovirus also attaches to sialic acid, with a strong preference for *O*-linked glycans containing sialic acid α2-3-linked to galactose [161]. Differences in receptor specificity appear to be virulence markers of TMEV. While strains with high neurovirulence bind to heparan sulfate, low

neurovirulence strains bind to α2-3-linked sialic acid moieties on *N*-glycans [162]. Crystallographic studies revealed a positively charged area on the viral surface in contact with sialic acid through non-covalent hydrogen bonds to be important for the persistent infection of the non-neurovirulent strain [158].

7 Reoviridae

These viruses have a segmented double-stranded RNA genome that is encapsidated by one, two, or three protein layers. The icosahedral virions are not enveloped and have a diameter of about 80 nm. There are 12 genera in the virus family. Binding to sialic acid has been observed with many members of the *Rotavirus* genus (for references see below), and some viruses belonging to serotypes 1 and 3 of the *Orthoreovirus* genus [163] also recognize such receptors.

Orthoreoviruses occur with a variety of vertebrates. Infection in humans is generally benign, but may cause upper respiratory tract illness and possibly enteritis in infants and children. Infection is initiated by receptor binding of the sigma 1 protein located in the outer layer of the viral capsid. Sigma 1 forms trimers and is composed of a fibrous tail containing the sialic acid-binding site and a globular head domain that interacts with junctional adhesion molecule 1 (JAM-1) serving as a secondary receptor [164, 165]. The ability of the sigma 1 protein to bind to sialic acid depends on a point mutation (L204P) at the binding site that converts a sialic acid-negative into a sialic acid-positive binding phenotype [164]. Interaction with sialic acid appears to precede binding to JAM-1 and to be necessary for endocytosis of the virus [166].

Rotaviruses infect a wide range of avian and human species and they are the major cause of gastroenteritis in children. Virions possess an outer VP7 layer and large "spikes" or "turrets" at the 12 icosahedral vertices composed of VP4. Trypsin cleaves the C-terminal from the N-terminal domain of VP4, giving rise to VP5 and VP8, respectively, both of which remain associated with the virion. X-Ray crystallography and NMR spectroscopy of VP8 alone and complexed with 2-0-methyl-α-D-*N*-acetyl neuraminic acid revealed that the VP8 core is a globular domain of an 11-stranded anti-parallel β-sandwich with the sialic acid binding site located in an open-ended, shallow groove [167, 168]. The concept that rotaviruses attach to sialic acid is supported by the observation that binding of some strains to cells is abolished by neuraminidase treatment [169, 170]. In contrast, binding of many other strains is neuraminidase insensitive [171], but it is now clear that these viruses also use sialic acid, yet in a form resistant to neuraminidase treatment [172, 173]. Comparison of the crystal structures of VP8 of neuraminidase-sensitive and neuraminidase-insensitive strains revealed that they were very similar, differing only by the size of the sialic acid binding groove that was slightly wider with the neuraminidase-insensitive strain [174].

The following steps are believed to be involved in the cell entry of rotaviruses: The VP8 domain of VP4 binds first to sialic acid residues of gangliosides or

glycoproteins resulting in a conformational change of VP4 that exposes VP5. The VP5 domain then interacts with α2β1 integrin. Finally, several additional interactions take place, involving VP5, VP7, integrins αvβ3 and αxβ2, and probably other cellular proteins [175]. Compatible with this concept is the observation that rotavirus binding to sialic acid is characterized by broad specificity and low affinity, suggesting that it mediates initial cell attachment prior to other interactions that determine host range and cell type specificity [176].

Different gangliosides have been found to be involved in rotavirus entry, and the results of these studies have recently been reviewed in detail [10]. Briefly, porcine rotaviruses have GM3 [177] and GD1a [173] receptors. Simian rotavirus 11 binds to GM3, GM2, and GD1a [178, 179]; GM3 containing both *N*-acetyl- and *N*-glycolyl-neuraminic acid may represent the receptors of bovine rotaviruses [178]. Human rotavirus bound to GM1 [173, 180].

8 Polyomaviridae

Polyomaviruses are DNA-tumor viruses. Most of them have oncogenic potential in rodents and non-human primates, and murine polyomavirus (MPyV) and simian polyomavirus 40 (SV40) have been widely used in experimental oncology. In immunocompromised patients, the human polyomaviruses JCPyV and BKPyV cause progressive multifocal leucoencephalopathy, a fatal demyelinating disease, and nephropathy, respectively. The recently discovered Merkel cell polyomavirus (MCPyV) is the causative agent of an aggressive form of human skin cancer. Polyomaviruses are small non-enveloped viruses containing a double-stranded DNA genome. VP1 is the major viral protein. It forms the outer capsid shell of the icosahedral virions and carries the receptor binding site [181–184].

Paulson and his group were the first to show that MPyV utilizes sialic acid as receptor. Employing reconstituted erythrocytes with defined sialic acid moieties they found that some strains specifically bound to α2-3-linked sialic acid, whereas others also recognized branched α2-6-linkages [185–187]. More recently, gangliosides GD1a and GT1b were identified as receptors in sucrose gradient floatation assays [188]. Crystallographic analysis has shown that a shallow groove composed of several loops of VP1 serves as the sialic acid binding site [184, 189]. The structural analysis also showed that the receptor pocket specifically accommodates a Neu5Acα2-3-Gal motif unbranched at the Gal position [183] which is compatible with the data obtained in the binding studies employing erythrocytes [186] and gangliosides [188]. MPyV also uses α4β1 integrin as receptor [190] which appears to be mediated by an LDV integrin binding groove deep within VP1. This suggests that, after attachment to sialic acid, the virus has to undergo a conformational change that allows binding to integrin as a second step in the entry process [8]. Evidence has also been obtained that binding to gangliosides promotes virus entry via caveolin-mediated endocytosis [191, 192].

SV40 also binds to gangliosides, but it differs in its receptor specificity from MPyV by showing a specific requirement for GM1 [188]. Crystallographic analysis has revealed that both the Galβ1-3GalNAc and Neu5Ac branches provide binding activity by directly contacting the protein [182]. Receptor binding of African green monkey lymphotropic papovavirus (LPV), another primate polyomavirus, has been shown to be neuraminidase sensitive, and it has been suggested that the sialic acid necessary for the receptor function is located on a mucin-type glycoprotein or on a ganglioside [193].

Knowledge on the receptors of the human polyomaviruses is less detailed. JCPyV binds to α2-3- and α2-6-linked sialic acid [194, 195], and there is some evidence that ganglioside GT1b is involved in the infection of human neuroblastoma cells [196]. Infection of glial cells depends on the serotonin receptor 5HT2a, and this receptor function appears to be neuraminidase sensitive [195, 197]. BkPyV binds only to α2-3-linked sialic acid [198], and floatation assays have shown that gangliosides GD1b and GT1b serve as receptors [199]. GT1b was also identified as a receptor of MCPyV, and the observation that GD1a and GD1b did not show this function suggests that both the α2-3-linked and the α2-8-linked sialic acid of GT1b are required [200].

9 Adenoviridae

This family contains non-enveloped DNA viruses that bind to their receptors via interactions with the distal knob of the penton fibers attached to the vertices of the icosahedral virions. Human adenoviruses mainly cause respiratory and gastrointestinal infections. Several adenoviruses also infect the eye where the most important disease is epidemic keratoconjunctivitis (EKC), caused primarily by Ad8, Ad19, and Ad37. Ad37 binds preferentially to α2-3-linked sialic acid which is the most frequent type of sialic acid linkage in corneal and conjunctival cells [201]. The crystal structure of the Ad37 knob–sialic acid complex has been elucidated [202].

10 Parvoviridae

This family contains small icosahedral viruses with a single-stranded DNA genome that is encapsidated by a shell composed of two or three proteins. The *Parvoviridae* family is subdivided into two subfamilies (*Parvovirinae* and *Densovirinae*) comprising a total of nine genera, two of which contain viruses that recognize sialic acid receptors. These are the minute virus of mice in the *Parvovirus* genus and some adeno associated viruses (AAVs) in the *Densovirus* genus. AAVs are non-pathogenic agents that depend on adenoviruses for replication. Because of their inability to induce productive infection in the absence of a helper virus, AAVs are promising vectors in gene therapy.

Bovine AAV has been shown to depend on gangliosides for entry [203], and binding to α2-3-linked sialic acid has been reported for AAV type 5, whereas AAV4 appears to bind to α2-6-linked sialic acid [204]. It has also been suggested that sialic acid serves not just as an attachment factor but is also required for virus internalization [205]. On the whole, however, the role of sialic acid in the AAV infection process is still poorly understood.

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