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MINIREVIEW

Role of sialic acid-containing molecules in paramyxovirus entry into the host cell: A minireview

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Abstract Sialic acid-containing compounds play a key role in the initial steps of the paramyxovirus life cycle. As enveloped viruses, their entry into the host cell consists of two main events: *binding* to the host cell and *membrane fusion*. Virus adsorption occurs at the surface of the host cell with the recognition of specific receptor molecules located at the cell membrane by specific viral attachment proteins. The viral attachment protein present in some paramyxoviruses (Respirovirus, Rubulavirus and Avulavirus) is the HN glycoprotein, which binds to cellular sialic acid-containing molecules and exhibits sialidase and fusion promotion activities. Gangliosides of the gangliotetraose series bearing the sialic acid *N*-acetylneuraminic (Neu5Ac) on the terminal galactose attached in α2-3 linkage, such as GD1a, GT1b, and GQ1b, and neolacto-series gangliosides are the major receptors for Sendai virus. Much less is known about the receptors for other paramyxoviruses than for Sendai virus. Human parainfluenza viruses 1 and 3 preferentially recognize oligosaccharides containing *N*-acetyllactosaminoglycan branches with terminal Neu5Acα2-3Gal. In the case of Newcastle disease virus, has been reported the absence of a specific pattern of the gangliosides that interact with the virus. Additionally, several works have described the use of sialylated glycoproteins as paramyxovirus receptors. Accordingly, the design of specific sialic acid analogs to inhibit the sialidase and/or receptor binding activity of viral attachment proteins is an important antiviral strategy. In spite of all these data, the exact nature of paramyxovirus receptors, apart from their sialylated nature, and the mechanism(s) of viral attachment to the cell surface are poorly understood.

Keywords Viral receptors · Paramyxovirus · Gangliosides · Sialidase · HN glycoprotein

Abbreviations

DANA	Neu5Ac2en 2,3-dehydro-2-deoxy-*N*-acetyl neuraminic acid
FANA	deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid
4-GU-DANA	2,3-didehydro-2,4-dideoxy-4-guanidino-N-acetylneuraminic acid
SV5	simian virus 5
hPIV1, 2 or 3	human parainfluenza virus 1, 2 or 3
NDV	Newcastle Disease Virus
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
α-Neu5thioAc2Sme	α-2-S-methyl-5-N-thioacetylneuraminic acid
RSV	Respiratory Syncitial Virus

The authors would like to dedicate this review to Prof. José A. Cabezas, recently retired who, as well being our mentor and colleague, introduced us into the fascinating field of sialic acid-containing glycoconjugates and viral sialidases at a time when just a very small number of scientists were paying attention to this important field of research. Also, he has been for us a continuous source of inspiration and friendship to us.

The ganglioside nomenclature of Svennerholm [1] is used.

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1. Introduction

Viruses are unable to replicate on their own and they are obligate parasites of all types of living cells, including some microorganisms, this relationship with their host being a key role to their survival [2]. This has forced viruses to develop a large repertoire of strategies for entering into the host cell. Viruses and cells have deep evolutionary roots and an ancient origin of viruses over a long process of coevolution with cells has been suggested [3]. Viruses also have the capacity to exploit alternative entry pathways, reflecting their role as agents for horizontal gene transfer among cells [4]. This coevolution enabled both to share many important functional macromolecular machinery and mechanisms [5]. Virus entry into the host cell consists of two main events: *binding* and *penetration* [6]. Therefore, virus-host cell interactions start with the binding of the virus to the host cell surface, a process that must be stable enough to ensure efficiency. This is accomplished by the recognition of specific receptor molecules located at the surface of the plasma cell membrane by specific viral proteins (viral attachment proteins). Also, in several cases one receptor molecule (usually broadly distributed) is required for virus-cell binding, while a second molecule, called the *coreceptor*, is required to trigger penetration. Both terms, receptor and coreceptor, are usually referred to as cell surface factors that bind to native virions. Additionally, the tissue distribution of receptors (and coreceptors) in part determines the symptoms of infection. Since receptors and coreceptors are major determinants of viral tropism, limiting the host range, they have been the object of intensive research. Precise knowledge of the nature of the viral attachment proteins and of receptors and coreceptors may help to develop new antiviral and vaccine strategies.

Some viruses, such as the paramyxoviruses, are composed of a nucleocapsid surrounded by an outer *envelope* consisting of a lipid bilayer with integral glycoproteins. Therefore, the entry of these viruses into cells requires the fusion of viral and cellular membranes, a process driven by viral proteins. Enveloped virus entry into the host cell thus consists of two main events: *binding* and *membrane fusion*. To establish efficient virus-cell interactions leading to a successful infection cycle, viral membrane proteins of enveloped viruses must therefore carry out at least two different functions, some of them combined in a single protein or others residing in different polypeptides: (i) *receptor binding activity* to specifically attach the virus to the host cell surface, making virus release an unlikely event; (ii) *membrane fusion activity* to deliver the viral genome into the cell cytoplasm. Also, in some cases there is a third activity, *receptor-destroying activity,* to promote viral spread; indeed, viral tropism also depends on the pathway of spread in the infected host.

The *Paramyxoviridae* forms a family of enveloped, negative-stranded RNA viruses that cause diseases in humans and animals [reviewed in 7]. This family of viruses has recently been reclassified into two subfamilies: the *Paramyxovirinae*, containing five genera, Respirovirus, Rubulavirus, Morbillivirus, Avulavirus and Henipavirus, and the *Pneumovirinae*, containing two genera, Pneumovirus and Metapneumovirus (Table 1). Among the human disease-causing paramyxoviruses are measles, parainfluenza, respiratory syncytial, mumps viruses, and the Hendra and Nipah viruses. Other paramyxoviruses infect animals, some of them used in human nutrition or in close contact with humans (such as horses), and they may have important economic impacts: Newcastle disease, Hendra, Nipah and rinderpest viruses. Paramyxoviruses have a non-segmented negative-stranded RNA genome of 15,000 to 19,000 nucleotides in length, bound to three different proteins: NP, P and L. The RNAs of paramyxoviruses are tightly bound to the NP protein and they are also associated with the P and L proteins. Together, these components constitute the ribonucleoprotein complex, which is the active template for the transcription and replication of the viral genome.

The outer envelope of paramyxoviruses consists of a lipid bilayer containing two integral glycoproteins and a non-glycosylated protein, the M (matrix) protein, located in virions beneath the inner lipid leaflet. Some rubulaviruses and all pneumoviruses have a third integral membrane glycoprotein. All integral glycoproteins are inserted to the membrane by an anchor segment of their polypeptide and project most of their structure to the outside, forming the so-called viral "spikes". In these glycoproteins reside the above-mentioned essential activities for viral entry and spread: receptor binding, membrane fusion and receptor-destroying activities. Viral attachment proteins from paramyxoviruses, bearing receptor-binding activity, come under several names, depending on the virus: HN, H or G proteins. A different protein is responsible for viral-host cell membrane fusion: the fusion (F) protein [8]. It is possible that, unlike orthomyxoviruses, the separation of such viral properties (receptor binding and fusion) on separate proteins may facilitate the modification of receptor recognition without compromising the triggering of fusion [9], thus explaining the broad variety of hosts that paramyxovirus are able to infect.

It is well established that the replication cycle of paramyxoviruses takes place in the cytoplasm of the host cell (reviewed in [7]). Virus adsorption occurs at the surface of the host cell membrane. The viral attachment protein (HN, H or G, depending on the virus) recognises and binds to sialic acid-containing molecules such as glycoproteins and glycolipids. Once the virus has attached itself to the host cell membrane, the viral F protein promotes the fusion between the viral and cellular membranes and the nucleocapsid is delivered into the cytoplasm. The membrane fusion

Table 1 Attachment proteins and putative receptors for some members of the *Paramixoviridae* family

Virus	Attachment protein	Receptor	Attachment cofactors	References
Genus Respirovirus	HN			
Sendai virus		Gangliotetraose-series gangliosides bearing Neu5Ac on the terminal galactose attached in α2–3 linkage and neolacto-series gangliosides; glycophorin and GP-2 glycoproteins		[88,90–95,87,97,98]
			ASGP-R; Heparin	[100,101,106,108]
hPIV1		Oligosaccharides containing *N*-acetyllactosaminoglycan branches with terminal Neu5Ac∀ 2-3Gal		[93]
hPIV3		Oligosaccharides containing *N*-acetyllactosaminoglycan branches with terminal Neu5Ac ∀ 2-3Gal; Neu5Ac ∀ 2-6Gal- or Neu5Gc∀ 2-3Gal- containing receptors		[93]
			Heparan sulfate	[105]
Genus Avulavirus	HN			
NDV		Linear lacto-series oligosaccharides and GM3, bearing both Neu5Ac or Neu5Gc sialic acids; different gangliosides as GM3, GD1a and GT1b, GM2, GM1 or GD1b; *N*-Glycoproteins		[62,92]
Genus Rubulavirus	HN	Sialylated molecules		
SV5				
Mumps virus				
PIV2				
PIV4				
Genus Morbilivirus	H			
Measles virus		CD46 and SLAM (CD150)		[120–122]
Canine distemper virus		Molecular homologous to human SLAM		[124]
Rinderpeste virus		Molecular homologous to human SLAM		[124]
Genus Pneumovirus	G			
RSV		Glycosaminoglycans		[82,83,117–119]
Genus Henipavirus	G			
Hendra virus		Surface protein		[126]
Nipah virus				

process takes place at the host plasma membrane and is pH-independent. However, it has been reported that Newcastle disease virus (NDV) may also penetrate the cell following the endocytic pathway in a pH-dependent process [10]. The RNA of paramyxoviruses contains all the genes encoding the structural proteins. Transcription is guided by short, conserved sequence motifs that flank each gene, the gene-start signal and the termination/polyadenylation or gene-end signal. After synthesis, viral structural glycoproteins undergo folding and conformational maturation, assisted by folding enzymes and molecular chaperones (reviewed in [7]). Only correctly folded and assembled glycoproteins are transported to the endoplasmic reticulum and Golgi apparatus, where they are post-translationally modified, a key process involved in gaining their biological activities. These post-translational events include glycosylation (*i.e.* [11–16]) and disulfide formation, leading to their oligomerization (*i.e.* [17,18]). The assembled ribonucleocapsid interacts with the M protein and the inner leaflet of the host cell plasma membrane. Also, the M protein interacts with the cytoplasmic tail of the membrane-bound viral glycoproteins [19]. Like other enveloped viruses, paramyxoviruses are formed by a budding process, emerging from sites on the host plasma membrane, where viral components (ribunucleocapsids and membrane-bound proteins) assemble and pinch off, resulting in the release of a new progeny of virions. In this process, the M protein plays a crucial role by binding to the ribonucleocapsids and to the inner lipid leaflet of the host cell plasma membrane, where integral viral enveloped glycoproteins have been inserted (recently reviewed in [20]).

2. Viral attachment proteins

The receptor binding activity of all paramyxoviruses (reviewed in [7]) resides in a single membrane-bound glycoprotein. In most cases, receptor molecules are glycoconjugates of different nature. The attachment glycoprotein present in Respirovirus, Rubulavirus and Avulavirus (Table 1) binds to cellular sialic acid-containing receptors that may be sialoglycoproteins and/or sialoglycolipids in a process that is believed to be of low affinity, but strong enough to make detachment an unlikely event. This protein is also able to agglutinate erythrocytes (hemagglutination) and exhibits sialidase (=neuraminidase) activity, hence its designation: HN protein. In the case of Morbillivirus, the attachment glycoprotein has hemagglutinating activity, but lacks sialidase or esterase activities, being designated H protein. Pneumoviruses have an attachment protein with no hemagglutinin or sialidase activities and this is designated protein G (Table 1).

HN protein

The HN protein is present in Respirovirus (i.e. Sendai virus), Rubulavirus (i.e. Mumps virus, Simian virus 5 or SV5), and Avulavirus (NDV) and binds to sialic acid-containing molecules. It is a multifunctional glycoprotein and the major antigenic determinant of the paramyxoviruses. The protein has three main biological activities with important roles in the viral infection cycle: hemagglutinin, sialidase and fusion promotion activities. The hemagglutinin allows the binding of the virus to the host cell surface by recognizing specific cellular receptors. The sialidase facilitates the viral spread of the newly born virions. Finally, the fusion promotion activity enhances viral F protein fusion activity.

Viral sialidase activity hydrolyses sialic acid residues from sialylated glycoconjugates present in the host, and the virus will not remain attached to host sialylated soluble glycoproteins or to host secretions that the virus may encounter before facing its true host cell [21,22]. Additionally, cellular receptors may be incorporated in the viral envelope at the time of budding and it has been suggested [23] that their presence may elicit the premature release of viral F protein fusion peptide, preventing virus-cell membrane fusion. According to this hypothesis, HN sialidases would facilitate viral entry by destroying cell receptors on the viral envelope, thereby preventing indiscriminate F activation [23]. However, it has been described [24] that the NDV envelope has GD1b and GT1b gangliosides, molecules that are considered to be NDV receptors (see later). This means that the sialidase does not remove all sialic acid residues from the sialylated glycoconjugates present in the viral envelope, although this does not prevent the virus from spreading. It is therefore possible than an effective removal of sialic acid residues from the viral surface would not be necessary for viral spread.

Fusion promotion activity was first described for NDV [25–27] and hPIV3 [28]. There are many other works favouring the existence of this activity specifically related to homotypic HN. Data on hPIV2, hPIV3 and NDV suggest the direct involvement of the HN protein in the promotion of fusion [13,28–32]. Furthermore, some antibodies raised against HN affecting fusion promotion activity do not interfere with receptor-binding or sialidase activities [33–35].

The HN polypeptide chain ranges from 565 to 582 residues (reviewed in [7]). This is a class II integral membrane protein that crosses the membrane only once and has its N-terminus sequence inserted in the lipid bilayer, but exposing a short N-terminal peptide, called the cytoplasmic tail, to the outside of the inner lipid leaflet. Most of the HN polypeptide including the *C*-terminal end is exposed to the outside of the outer viral lipid leaflet, forming an extracellular hydrophilic domain, the "spike" [36], where the receptor-binding, sialidase and fusion promotion activities are located in the ectodomain, consisting of a globular domain and a stalk region close to the outer lipid leaflet. The structure of the ectodomain displays a six-bladed β-propeller fold typical of other known sialidases [37–43]. HN is usually present as a tetrameric structure, consisting of a dimer of two disulfide-linked homodimers [42]. However, it has been shown that the HN soluble ectodomain of the NDV Clone-30 strain behaves as a monomer, but has a lower activation energy for sialidase activity, suggesting the importance of the transmembrane segment of the protein and its insertion into the membrane in the overall structure and function of the glycoprotein [44].

The topology of the receptor-binding and sialidase activities within the HN ectodomain is still a very controversial issue. The first report known in paramyxoviruses concerning the existence of sialidase and hemagglutinin activities present in the same protein comes from SV5 [45]. In the literature, authors have proposed the existence of two independent sites; others the existence of a single one and some papers have even proposed two sites in close proximity, but current knowledge favours the existence of two independent active sites. Previous work performed by means of monoclonal antibody binding data, lipid environmental effects on HN activities, and mutant studies favoured the existence of two separate sites [*i.e.* 24,46–53] but failed to provide information about the precise location of both sites. Fortunately, the crystal structure of the NDV HN globular ectodomain, obtained by crystallization of the protein in a complex with the sialidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en), has been determined [42] and the sialidase active site was found to be located within a β-propeller fold. Additionally, a recent

study [54] has located a second sialic acid-binding site at the membrane-distal end of the dimer interface, uncovered by cocrystallization of HN with the non-hydrolyzable substrate thiosialoside Neu5Ac-2-5-α(2,6)Gal1OMe, in the NDV HN globular domain of the protein. Mutations at this second binding site did not affect sialidase activity, although fusion promotion activity was abolished. Additionally, the importance of the dimer interface in receptor-binding activity seems to be clear, since mutations in this domain prevent receptor binding [55]. Also, in a recent work gangliosides acting as receptor mimics did not compete with Neu5Ac for binding, although both elicited a conformational change in the HN glycoprotein [56]. Recently, mutational and kinetic studies on NDV HN glycoprotein [32] have revealed that single mutations made in some residues belonging to the sialidase active site affect sialidase and receptor-binding activities to different extents. The discrepancy in the modifications in sialidase and receptor-binding activities in the single mutants analysed does not account for the topological coincidence of the two sites. Similarly, recent studies concerning the mode of action of the sialic acid analog 2,3-didehydro-2,4-dideoxy-4-guanidino-N-acetylneuraminic acid (4-GU-DANA) on the inhibition of NDV and hPIV3 HN receptor-binding activity [57] also support the existence of two separate active sites in NDV HN, one with both sialidase and receptor binding activity, and the second one with only receptor-binding activity.

The location of HN fusion promotion activity has not been completely elucidated. A considerable body of evidence using chimeras and mutant HNs suggests a topological separation of sialidase and hemagglutinin activities from fusion promotion activity, pointing to its location in the transmembrane or the stalk domains of the protein [*i.e.* 26,32,51,58–62]. However, other data point to the participation of the globular ectodomain [63]. Some recent data using HN mutants in the globular ectodomain suggest that this domain does not participate in fusion promotion activities [64] and that the observed lower fusion activity would be due to a modified receptor binding capacity. However, other studies [32] describing mutants not affected in their binding capacity but with lower fusion activity suggest a direct role of the globular ectodomain. Further investigation must be conducted to clarify this topic.

H protein

This attachment protein is present in viruses belonging to the genus Morbillivirus, whose best-known representative is measles virus (Table 1). Other viruses belonging to this genus are canine distemper and rinderpest viruses. The H protein is able to agglutinate erythrocytes and, in the case of measles virus, lacks sialidase or esterase activities. However, a sialidase activity of the H protein from rinderpest virus has been described [39]. Protein H is also a type II transmembrane glycoprotein. It acts as an important determinant of Morbillivirus tropism [65] and cooperates with the F protein in the membrane fusion process (reviewed in [66]). Interestingly, the structure of the hydrophilic ectodomain displays the typical bladed β-propeller fold found in the HN protein [39]. As in the case of other paramyxovirus glycoproteins, the H protein is also glycosylated, folded and oligomerized by disulfide formation in the endoplasmic reticulum before being transported to the host plasma cell membrane [66]. Glycosylation is essential for proper folding and intracellular transport from the Golgi. In the binding of protein H to its receptor 18C-terminal residues are involved ([67], and reviewed in [66]).

G protein

The attachment protein of paramyxoviruses of the genus *Pneumovirus* [68], which is usually represented by the respiratory syncytial virus (RSV), is the so-called protein G. Surprisingly, however, it has been shown that the G protein is not required for infectivity, at least in cultured cells [69]. Nevertheless, it is believed that although G is dispensable *in vitro* it is important for efficient infection *in vivo* [70]. The G protein lacks both hemagglutinin and sialidase activities and is structurally different from the attachment proteins (HN or H) of other paramyxoviruses (reviewed in [70]). The G protein is also a type II integral membrane protein, having its *N*-terminal end inserted into the lipid bilayer and exposing a hydrophilic ectodomain. The G protein is synthesized as a precursor that undergoes extensive *N*- and O-glycosylations [71] in a manner dependent upon the viral strain and cell type [72,73]. Thus, protein G is similar to mucins [74], the glycoproteins forming a barrier in the respiratory tract. In the mature protein, the ectodomain contains two mucin-like domains [75]. Glycosylation occurs cotranslationally and later the protein assembles into oligomers. RSV G protein binds to cellular glucosaminoglycans [76,77] (see below).

3. Sialic acid-containing glycoconjugates: Receptors for paramyxoviruses

As mentioned above, the virus receptor is defined as a molecule or molecules present at the host cell surface that are specifically recognized by the virus; after virus-receptor binding, viral infection of the cell may start [78]. Although it is well established that most paramyxoviruses infect cells in a sialic acid-dependent manner [79], the exact nature of paramyxovirus receptors, apart from their sialylated nature, and the mechanism of viral attachment to the cell surface are not well known. It is thought that both sialoglycoproteins

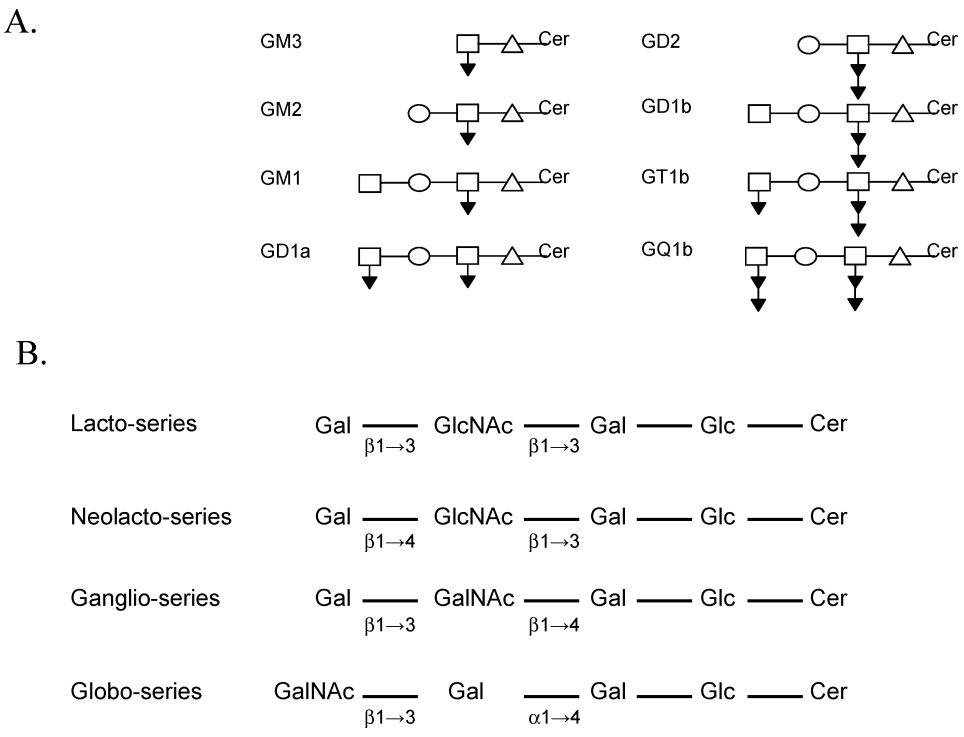

Fig. 1 (A) Chemical structure of gangliosides. Cer, ceramide; △, glucose; □, galactose; O, *N*-acetyl galactosamine; ▼, *N*-acetylneuraminic acid. (B) Core structure of the sugar chain of different series of gangliosides

[80,81] and gangliosides [78,80–83] may serve as viral receptors, although paramyxoviruses do not bind indiscriminately to any sialic acid-containing molecules present at the cell surface.

Gangliosides (glycosphingolipids having a hydrophilic oligosaccharide chain containing one or more sialic acid residues bound to a hydrophobic ceramide, which anchors the glycoconjugate to the membrane) and glycoproteins are potential receptors for most members of the *Paramixoviridae* family. The sialic acid motif in the receptor of paramyxoviruses was proposed many years ago. A considerable body of work carried out in the 70's and 80's reported the interactions *in vitro* of different paramyxoviruses to glycoproteins, such as serum glycoproteins, or to different gangliosides (for a review see [78]). Whether both types of compound serve as receptors at the cell surface to promote productive infection by the viruses and, additionally, whether both play similar roles in the virus-cell surface interaction multistep process (see below) are issues that remain to be solved.

Unlike bacterial sialidases, which remove a broad spectrum of sialic acid-containing molecules, the sialidase activity of paramyxoviruses is more restrictive. HN proteins from different paramyxoviruses completely release the sialic acid attached in a α2-3 linkage, but are inactive against substrates containing α2-6-linked sialic acid residues [references in 79]. In addition, sialic acid residues attached in a α2-8 linkage to another sialic acid molecule may be hydrolyzed by viral sialidases.

The recognition of sialic acid-containing molecules as viral receptor(s) depends on several factors [79]: (1) The type of sialic acid residue, mainly Neu5Ac or Neu5Gc; (2) the linkage between the sialic acid residue(s) and the oligosaccharide chain; (3) The sequence of the oligosaccharide chain; (4) The number of sialic acid residues, and (5) The spatial arrangement of the sialic acid residues. Most of the information concerning the nature of the paramyxovirus receptor(s) arises from the murine parainfluenza Sendai virus (Table 1). A large number of studies [82,84–87 and reviewed in 78] have demonstrated that gangliosides (see Fig. 1) of the gangliotetraose series bearing the *N*-acetylneuraminic sialic acid (Neu5Ac) on the terminal galactose, such as GD1a, GT1b, and GQ1b, and neolacto-series gangliosides, are the major receptors for Sendai virus [17,83,85–88]. Nevertheless, this virus does not recognize gangliosides lacking the sialic acid on the terminal galactose, such as GM1 or GD1b [82]. Regarding the specificity of sialic acid linkage, Sendai virus recognizes gangliosides with α2-3 linkage, but not α2–6 linkage unlike several influenza strains [89]. For NDV, preliminary works in the 50's and 70's determined that the virus interacts with sialoglycoconjugates in the Neu5Ac form (revised in [78]). Using asialoerythrocytes coated with different gangliosides, Suzuki *et al.* [86] reported that the receptor determinants for NDV were linear lacto-series oligosaccharides and GM3, bearing both Neu5Ac or Neu5Gc sialic acid residues (see Fig. 2 for sialic acid structures). Nevertheless, in thin-layer chromatographic binding assays it has recently been reported [56] that NDV interacts

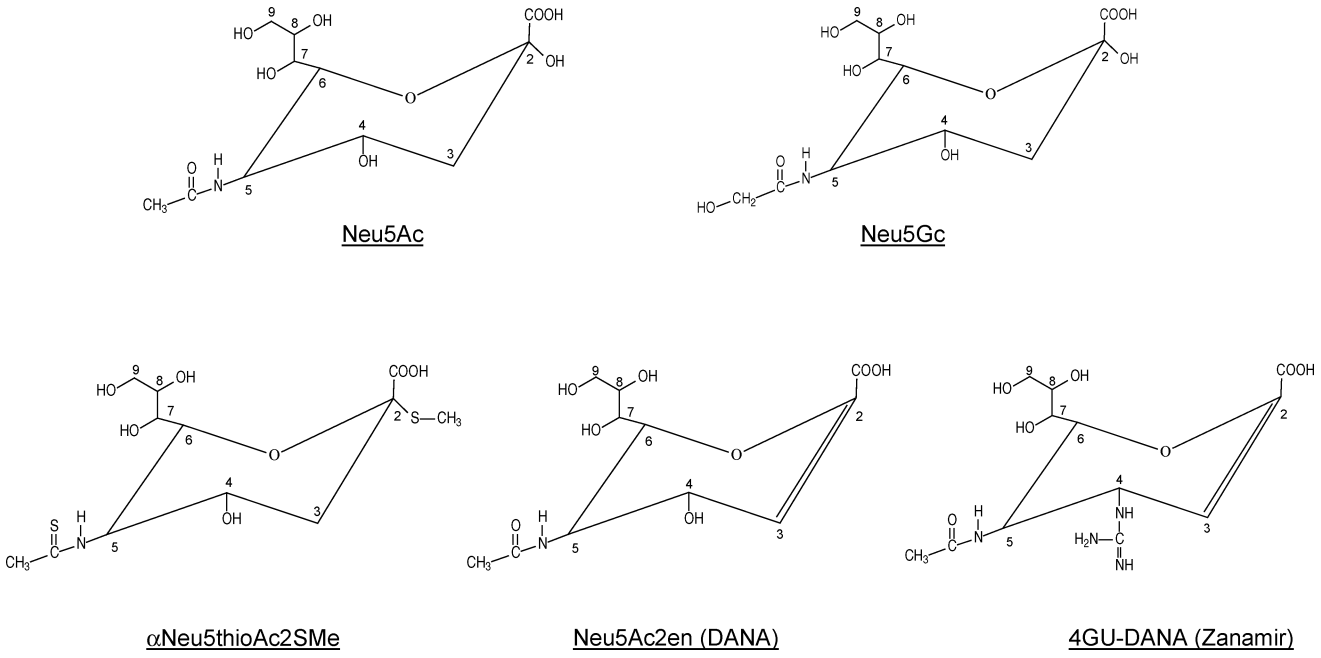

Fig. 2 Chemical structure of Neu5Ac and Neu5Gc sialic acids and of the synthetic sialic acid analogs as hypothetical inhibitors of Paramyxovirus entry

with different monosialogangliosides, disialogangliosides, and trisialogangliosides. In addition, NDV recognizes gangliosides with sialic acid residues attached to terminal sugars, such as GM3, GD1a and GT1b, or sialic acid attached to internal sugars, as in GM2, GM1 or GD1b. These data seem to point to the absence of a specific pattern of the gangliosides that interact with NDV. Accordingly, it seems that unlike Sendai virus the terminal sialyl linkage is not a requirement for NDV receptors [56].

The binding specificity of human parainfluenza viruses types 1 and 3 has recently been characterized [87]. The sialic acid-containing molecules recognized by hPIV1 are more limited than those recognized by hPIV3, suggesting a variability of receptor specificities that will depend on the different subtypes of viruses. hPIV1 and hPIV3 preferentially recognize oligosaccharides containing *N*-acetyllactosaminoglycan branches with terminal Neu5Acα2-3Gal, and hPIV3 also recognizes Neu5Acα2-6Gal- or Neu5Gcα2-3Gal- containing receptors. In contrast to Sendai virus, hPIV1 and hPIV3 do not bind to ganglio-series gangliosides, but they do recognize the terminal sialic acid residues and the type of the sugar chain. Since hPIV1 and hPIV3 bind to blood group I-type gangliosides with terminal Neu5Acα2-3Gal, these compounds could find potential use against parainfluenza infection [87] (see below). Orthomyxoviruses, like influenza viruses, also recognize sialic acid-containing glycoconjugates. Different influenza strains vary in their specificities as regards linkages (α2-3 or α2-6) and the type of sialic acid residue (Neu5Ac, Neu5Gc, or 9-O-Ac-Neu5Ac) (references in [90]).

Research into the role of glycoproteins in virus-cell binding has been less exhaustive. In addition to gangliosides, several studies have reported the use of sialylated glycoproteins as Sendai virus receptors such as glycophorin [91,92] or the GP-2 silalylglycoprotein [80,93]. In this sense, the fusion of Sendai virus with glycophorin-bearing liposomes has been shown to be as efficient as fusion with GD1a-containing liposomes [81]. In the case of NDV, we have recently reported [56] that N-glycosylated proteins at the cell surface are required for optimum viral fusion and entry, suggesting that both N-linked sialoglycoproteins and gangliosides may act as NDV receptors. In addition to the attachment function of HN proteins, several publications have speculated about the possible existence of a receptor for F protein [94–102]. The human asialoglycoprotein receptor (ASGP-R, a mammalian lectin) has been proposed as the attachment factor for Sendai F protein based on fusion studies of virus-like particles devoid of HN protein [94] or mutant viruses lacking the HN [95]. Additionally, reconstituted NDV envelopes devoid of HN protein show residual fusion actitivity, supporting the idea, among different hypotheses, of a receptor for F protein [96]. Unlike most paramyxovirus, SV5 may promote fusion in the absence of HN protein; this has been explained as a result of interaction of the F protein with a specific receptor of the target membrane before triggering of the fusion cascade, independently of the attachment function of HN protein [97]. Moreover, F protein of RSV may trigger fusion in the absence of the G attachment protein acting as F receptor heparin and heparan sulfate [98,99]. The interaction of Sendai virus with heparin has also been reported [100,101],

and, more recently, it has been shown that heparan sulfate also acts as a receptor for hPIV3 [102] although it is not clear whether it interacts with F or with HN protein. In this scenario, additional cell surface molecule(s) emerge as secondary or auxiliary viral receptors to sialic acid-containing receptor(s), as suggested for hPIV3 in the case of heparan sulfate [102]. For NDV, a mutant in the HR3 domain of the F protein [103] has been shown to fuse both with the sialic acid-defective cell line Lec2 [104] and in the presence of HN dimer interface mutants that show a minimal amount of attachment activity (see below). Since Lec2 cells retained 5–10% of the sialic acid residues from their parent cell line [105], these results were interpreted in terms of the mutant protein being capable of triggering fusion in the presence of low sialic acid-containing receptors, but in a sialic acid-dependent manner through an unknown mechanism [104].

Within this context, the interaction of viruses with the cell surface may be a complex scenario of interactions between the latter and the viral envelope during the virus entry process, governed by different cell surface molecules. For many enveloped viruses, it has been suggested that the viral attachment to the cell surface would be a multistep process [6]. It is possible that the virus receptor might consist of a complex of different cellular components, including glycolipids such as gangliosides, glycoproteins, and other proteins [106]. In this sense, it has been proposed that gangliosides may act as primary viral receptors [107–109] or as coreceptors [110–113]. This could be the case of paramyxoviruses, in which the virus may use different molecules with different roles in the virus-cell attachment process, *i.e.*, primary and secondary receptors [56]. Another possibility is that the virus might have the ability to use more than one receptor, depending on the host cell, since viral receptors are an important determinant of virus host range and tissue tropism. In influenza viruses a correlation between receptor specificities and host species of virus origin has been determined [87]. Further studies are required to dissect the role of different cell surface molecules (sialylated and non-sialylated) in virus entry into the host cell.

As mentioned, protein G is the attachment protein of the *Pneumovirus* RSV [68], the major cause of severe lower respiratory tract infection in infants and children. This mucin-like protein binds to heparin and chondroitin sulphate B, *i.e.*, glycosaminoglycans consisting of long unbranched polymers of repeating disaccharide units of glucuronic or iduronic acids, linked to glucosamine or galactosamine, with chemical modifications such as sulfation [76,77,98,99,114]. Iduronic acid seems to play a key role in the specificity of such binding [76]. It has also been suggested that the binding of the protein to glycosaminoglycans might merely be an initial contact, followed by a second more specific binding step [70]. More than one protein has been reported as the receptor(s) for the attachment H protein of the *Morbillivirus* measles virus: the CD46, a member of the regulators of the complement activation superfamily [115,116], and SLAM (or CD150), a signaling lymphocyte activation glycoprotein [117,118]. Molecules homologous to human SLAM have been reported to act as receptors for animal *Morbillivirus* such as canine distemper and rinderpest viruses [119]. Finally, Nipah virus and Hendra virus are members of the new genus *Henipavirus* [120], which includes novel paramyxoviruses from pigs and horses that are responsible for fatal zoonotic infections of humans. These viruses are most closely related to members of the genus *Morbillivirus* since their attachment G glycoprotein lacks both hemagglutinin and sialidase activities. Protease treatment of target cells completely abolishes Hendra virus-mediated fusion, suggesting that the virus must employ a cell surface protein as receptor [121].

4. Interactions of paramyxoviruses and sialic acid-containing receptors at the cell surface

In current models of paramyxovirus fusion [6,122], HN binds to the sialoglycoconjugate receptor(s) through its receptor-binding activity and undergoes a conformational change that, in turn, triggers a conformational change in the F protein to start membrane fusion (fusion promotion activity of HN protein). In this sense, a partial conformational change on NDV HN protein in the presence of free gangliosides acting as receptor mimics has been reported [123]. However, many questions remain unsolved: which part of the protein interacts with the receptor? What is the nature of the receptor-attachment protein interactions? Which changes on the protein are promoted after virus-receptor interaction?

As mentined above, based on crystallographic studies of the HN of NDV, two sialic acid-binding sites have been described [42,54,57], the active site located in a β-propeller structure, and the second binding site located at the dimer interface. The active site would support both sialidase and receptor-binding activity, and the second one only receptor-binding activity. The attachment of the viral protein to the cellular receptor through the second binding site, created after binding and catalysis of the active site [54], would hold the target membrane in close proximity with the viral membrane to increase fusion efficiency [54,58]. It remains unkown whether both sialic acid-binding sites might interact with the same cell surface molecule(s). Apart from the spatial localization of both HN activities, the receptor-binding and receptor-destroying activities of HN protein could be related at functional level, the balance between binding and receptor cleavage being crucial for viral entry. Based on experiments involving site-directed mutagenesis of conserved

residues at the predicted sialidase site of the globular domain of HN protein of NDV, it has been proposed that receptor binding would be completely dependent on sialidase activity [53]: critical sialidase residues are not essential for the attachment activity of the protein, although sialidase activity, of exogenous origin, is required for HN binding to cellular receptors [53]. One possibility is that breaking the association of HN with cell receptors would be necessary for HN to be released in order to mediate attachment. In principle, this would be opposite to the role proposed for the second sialic acid-binding site; *i.e.*, to maintain virus-cell proximity as fusion proceeds. A possible explanation that could be invoked to reconcile both hypotheses is that both HN sialic acid-binding sites might recognize different sialic acid molecules. Additionally, both sialidase and binding activities may be involved in the fusion promotion activity of the protein, such as, for example, modulating the level of receptors available for the virus and hence affecting the degree of cell fusion ([23] and references therein). In this sense, low levels of sialidase activity would favour fusion activity, as shown recently: a mutant of hPIV3 HN protein at the stalk and at the globular head devoid of sialidase activity was capable of triggering fusion, irrespective of pH. Nevertheless, the mutant with the same mutation only at the stalk domain showing 30% of wild-type sialidase activity was incapable of triggering F protein [23]. The residual sialidase of this mutant could fail to retain receptor contact long enough for F protein to become activated. Accordingly, it seems that the promotion of fusion of HN depends on the release of HN from the receptor through its sialidase activity [23].

5. Sialic acid analogs as antivirals agents

The key role of the HN protein in paramyxovirus infectivity means that, as a way of preventing paramyxovirus infection, the design of specific sialic acid analogs to inhibit its sialidase and/or its receptor-binding activity has emerged as an important antiviral strategy. Study of sialic acid analogs such as sialidase inhibitors began in the early 1970's with influenza HA protein as target. Compounds such as 2,3-dehydro-2-deoxy-N-acetyl neuraminic acid (Neu5Ac2en or DANA, see Fig. 2), a transition-state unsaturated derivative of sialic acid analog [124], and 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA) [125,126] were shown to be specific inhibitors of influenza sialidase. Recently, the sialic acid transition state analog 4-guanidino-Neu5Ac2en or 4-GU-DANA (also named Zanamir), designed on the basis of the crystal structure of influenza virus sialidase to fit its catalytic site, has been shown to be an effective anti-influenza agent used in the clinical treatment of influenza virus infections [127,128]. The analogs DANA and FANA and 4-GU-DANA also inhibit hPIV3 sialidase and receptor-binding activities [126,129,130]. Nevertheless, higher concentrations of 4-GU-DANA are required for inhibition of the enzymatic activity of paramyxoviruses [131] than that of influenza virus, making this compound unsuitable for the treatment or prophylaxis of infections caused by paramyxoviruses. Moreover, in the case of NDV, 4-GU-DANA inhibits the enzymatic activity of its HN but not its hemagglutinating activity [57]. Since crystallographic data from NDV [42] and hPIV3 HNs [132] are available, these structures may be useful models for the molecular design of specific inhibitors of paramyxovirus human and non-human pathogens. This is the case of two potent inhibitors of human parainfluenza viruses, BCX 2798 and BCX 2855, two derivatives of Neu5Ac2en, which were recently designed on the basis of the 3D structure of the HN protein [133]. In addition, the analog α-2-*S*-methyl-5-*N*-thioacetylneuraminic acid (α-Neu5thioAc2SMe), a sialic acid analog modified at C-2 and C-5 (Fig. 2), has been shown to inhibit the fusion of hPIV3 with cells and viral replication in cell cultures [1296]. Moreover**,** the crystallographic data of NDV glycoprotein complexed with Neu5Ac2en [42] indicate that analogs of Neu5Ac2en modified at C-4 might be useful for the development of anti-paramyxovirus agents. This is consistent with the reported inhibitory effect of hPIV1 sialidase exerted by eleven analogs of Neu5Ac2en modified at these two positions [134].

Outlook/concluding remarks

It is well established that sialic acid-containing compounds play a key role in the initial steps of the paramyxovirus life cycle. Unlike other related viruses such as Orthomyxovirus, the existence of two different glycoproteins for viral cell attachment and fusion functions, respectively, may confer this family of viruses with considerable variability with respect to the use of host cell receptors, which may account for the broad range of cells that are infected by these viruses and their success as pathogens [8]. Although the sialylated nature of paramyxovirus receptors is not controversial, the mechanism of HN-receptor(s) interaction is an issue still under current investigation, as well as the exact nature of the molecules that serve as receptor(s). The crystallographic data of several paramyxovirus HN proteins provide a basis for the development of sialic acid analogs aimed at blocking viral entry by interfering with the initial process of virus-cell surface interaction, *i.e.*, viral attachment and membrane fusion. In sum, HN proteins and sialic acid compounds arise as attractive targets for the design of structure-based drugs and sialic acid analogs for application in anti-paramyxovirus therapies aimed at fighting, at the cell surface, the entry of these viruses, which are major causes of respiratory diseases in infants and of huge economic losses.

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