Gangliosides

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Recognition of the GM3 Ganglioside Glycan by Rhesus Rotavirus Particles**

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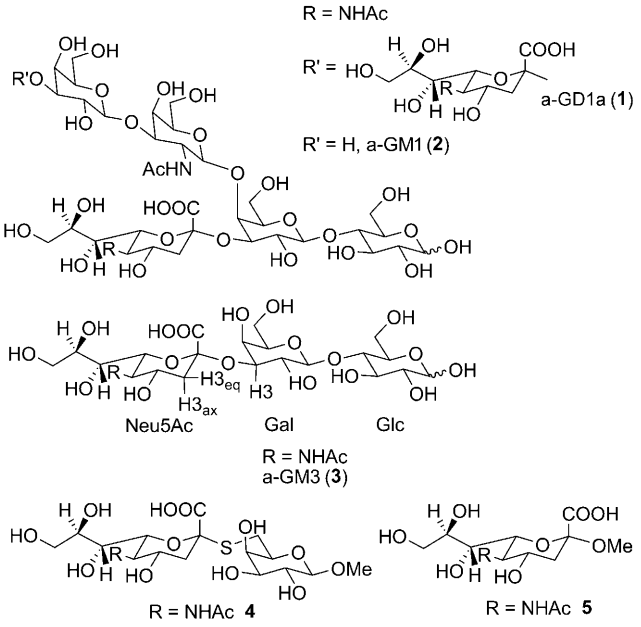
Rotaviruses are a major cause of severe infantile gastroenteritis in humans and animals worldwide, producing a childhood mortality exceeding 650000 annually.[1] Mapping host cell glycan–virus interactions to define a viral glycointeractome is invaluable in providing new directions for the discovery of novel broad-spectrum drugs and vaccines. In that context we have recently reported the first NMR-based structural analysis of the interaction of GD1a (**1**) and GM1 (**2**) ganglioside glycans with recombinantly expressed rotaviral surface lectin VP8* from two distinct rotavirus strains.[2]



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In that study we demonstrated the absolute requirement for sialic acid (Sia) and identified other subterminal carbohydrates, such as galactose (Gal), in host cell glycan–virus recognition mediated through the rotavirus VP8*. In the present work we have addressed a gap in our initial study and contribute to rotavirus glycobiology[3–5] with another important ganglioside rotavirus receptor, GM3. Furthermore, employing intact, infectious rotavirus particles, we also present our investigation on the influence of multiple copies of VP8* (higher protein valency) and trypsin activation[6,7] of virus on glycan recognition. Thus, herein we report saturation transfer difference (STD) NMR and cell-based experiments that reveal novel structural and functional insight into the interactions of the ganglioside GM3 glycan (**3**, a-GM3, Neu5Acα(2,3)Galβ(1,4)Glc) with Rhesus rotavirus (RRV) particles.

STD NMR spectroscopy[8,9] is an ideal tool to study the interaction between virus particles and ligands because the broad NMR signal linewidth of virions enables saturation without affecting ligand signals. A very limited number of STD NMR spectroscopic studies using intact virions or virus-like particles (VLPs), such as human rhinovirus,[10] H5-containing avian influenza VLPs,[11] and rabbit hemorrhagic disease VLPs,[12] have been published. A particular advantage of using whole virions or virus-like particles is that any contribution of the viral capsid environment in protein organization and function will be taken into account. This methodology provides a more biologically relevant model for the study of interactions between the virus and the host cell glycans.

The ^{1}H NMR spectrum of **3** (Figure 1a) and the STD NMR spectra of **3** when bound to RRV particles not treated with trypsin (Figure 1b) and trypsin-activated (Figure 1c) clearly reveals that RRV particles bind to **3**. Very strong STD NMR signals for the methyl protons of the *N*-acetyl group (NHAc, $\delta = 1.95$ ppm) are observed, and both trypsin-treated and untreated RRV particles bind to **3**. Furthermore, clear STD NMR signals are also observed for both the axial ($H3_{ax}$) and the equatorial protons ($H3_{eq}$) of Neu5Ac. A detailed comparison of these STD NMR spectra (Figure 1b and c) also reveals that the binding epitope of **3** when bound to trypsin-treated and untreated RRV particles is similar, if not identical. This important observation clearly suggests that trypsin activation of rotavirus particles is not essential for cell binding (adhesion) and therefore supports the widely accepted model that protease activation of virus is relevant for virus entry. Interestingly, a medium, but significant, contribution of the Gal moiety (H3 proton, $\delta = 3.85$ ppm) to the binding event is also detectable. This is consistent with our

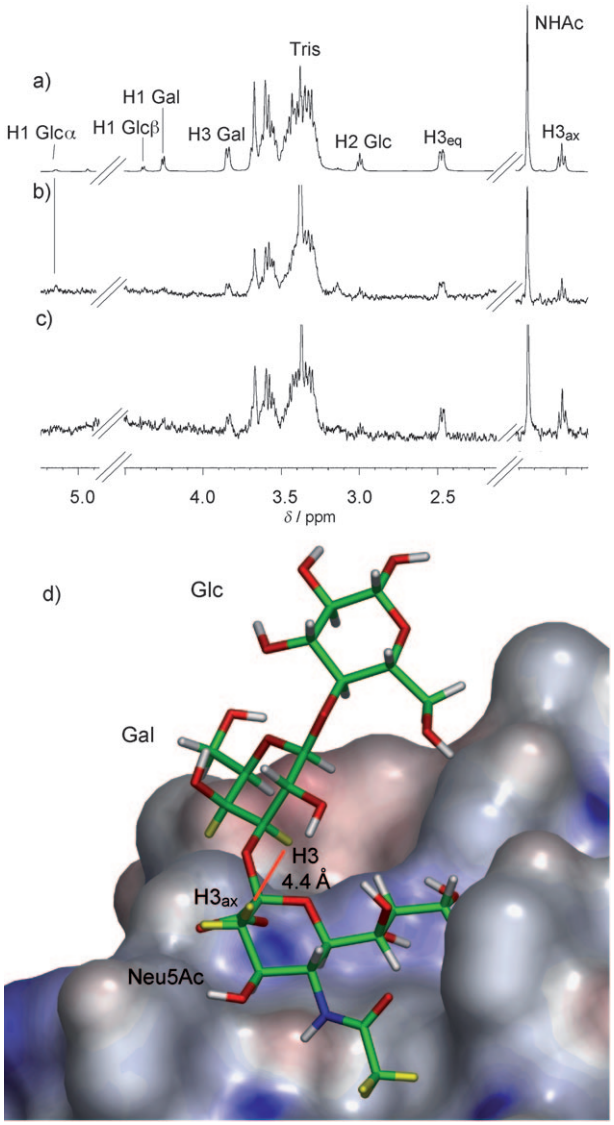

Figure 1. a) ^{1}H NMR spectrum of the a-GM3 ganglioside (**3**), b) STD NMR spectra of **3** in complex with untreated RRV particles, and c) STD NMR spectra of **3** in complex with trypsin-treated RRV particles at 600 MHz and 288 K. Tris = tris(hydroxymethyl)amino methane. d) A stick model of **3** complex with the binding pocket of RRV VP8* shows a large distance of 4.4 Å between the Gal H3 and Neu5Ac $H3_{ax}$ protons.

study[2] with more complex ganglioside glycans; it is in contrast to the previous notion that the aglycon unit, in general, does not play a role in the binding of sialosylglycosides to RRV VP8*.[13] In an earlier study we reported that the Neu5Ac moiety of the related thiosialoside **4** (Neu5Acα(2,6)-*S*-Galβ1Me), when bound to recombinantly expressed RRV VP8* protein, is involved in the majority of interactions with the protein, whereas the Gal moiety is predominantly solvent exposed.[3] The different interactions of the Gal residue of **3** and that in **4** with RRV VP8* can be explained in the context of the considerable dissimilarity between the α(2,3)- and α(2,6)-glycosidic linkages. The additional ω torsion angle in the case of the α(2,6)-glycosidic linkage substantially enhances flexibility, resulting in a binding epitope that is largely dominated by the sialic acid residue and sparse protein contact of the Gal moiety.[3] Additionally, the use of about 100 nm virus particles (ca. 74 MDa) in the current study compared to recombinantly expressed VP8* protein with a molecular weight around 18 kDa results in stronger saturation transfer caused by the larger correlation time of bulky virus particles and results in more efficient spin diffusion. Employing NOE and trNOE NMR spectroscopy, with a similar approach to that previously reported,[10] we have determined the bioactive conformation of the a-GM3 (**3**) Sia–Gal fragment in complex with VP8* using whole RRV particles and recombinantly expressed RRV VP8* (see the Supporting Information). We have also modeled (Figure 1d) **3** in the structurally characterized[5] VP8* binding site using our previously described methods (see the Supporting Information).[2] From these studies we have determined that the H3 protons ($H3_{eq}$ and $H3_{ax}$) and the methoxy protons of the *N*-acetyl group at C5 of the sialic acid, along with the H3 protons of the galactose units are all in proximity to the protein surface, in good agreement with our STD NMR data.

We have recently shown that the related CRW-8 rotavirus strain recognizes GD1a and not GM1. Moreover, we concluded that CRW-8 requires the terminal Sia moiety of a-GD1a (**1**) for efficient virus adhesion, and it appears to bind the internal Sia residue in a putative subterminal Sia binding site.[2] From the present work with **3** we have determined that the binding epitope of the Sia–Gal moieties of both gangliosides glycans **1** and **3** is identical. We hypothesize that the Sia–Gal moiety of a-GM3 ganglioside (**3**) binds to the high-affinity Sia binding site,[2] mimicking the terminal Sia–Gal moiety of the a-GD1a gangioside (**1**).

Based on the present and previously published data[2,3,13,14] we propose that although a range of sialic acids with varying linkage patterns can be recognized by the rotaviral VP8*, the higher affinity ligands are most likely gastrointestinal cell surface, multivalently displayed α-(2,3)-sialoglycoconjugates, particularly the gangliosides GM3, GM1, and GD1a. This is consistent with the fact that higher order gangliosides are in abundance in the intestinal brush border membrane.[15]

To further support our NMR data, RRV infectivity titers[16] in the presence of anti-GM3 antibody (GMR6[17]) were determined. This GM3 antibody significantly inhibited RRV infectivity, by $(30 \pm 5)\%$ at 20 $\mu g\,mL^{-1}$ ($p < 0.0001$) and by $(37 \pm 3)\%$ at 40 $\mu g\,mL^{-1}$ ($p < 0.0001$), suggesting that RRV particles and GMR6 antibodies compete for GM3-like receptor molecules (Figure 2a). Interestingly, cholera toxin B (CTB) did not significantly inhibit RRV infectivity at either 0.1 or 1.0 $\mu g\,mL^{-1}$ ($0.3 < p < 0.5$; Figure 2b) suggesting that RRV does not use GM1-like host cell receptors. These results are in excellent agreement with our previous study showing that the related CRW-8 rotavirus strain requires a terminal sialic acid moiety, as found on GD1a, for effective virion attachment and infectivity.[2]

The importance of the Gal moiety in **3** was further investigated by interrogating the binding epitope of methyl α-D-*N*-acetylneuraminide (Neu5Acα2Me, **5**) when bound to RRV particles. Figure 3a shows the ^{1}H NMR spectrum of **5** in complex with trypsin-treated RRV particles and Figure 3b

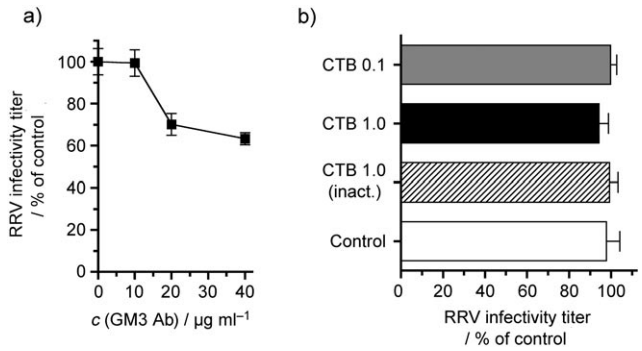

Figure 2. a) Inhibition of RRV rotavirus infection by addition of anti-GM3 antibody (Ab) GMR6. b) This antibody was not affected by addition of the GM1 ligand or *V. cholerae* toxin B subunit (CTB). Heat-inactivated CTB at 1.0 µg mL^{-1} (CTB 1.0 (inact.)) was included in (b) as a specificity control. Bar = standard deviation.

the corresponding STD NMR spectrum. The binding of **5** to the RRV virion demonstrates that the overall binding epitope is comparable to the binding epitope obtained for a complex of recombinantly expressed RRV VP8* and **5**[3] with a major involvement of the *N*-acetyl moiety (NHAc, $\delta = 1.85$ ppm) and simultaneously the less important role in binding of the aglycon methyl protons (OMe, $\delta = 3.15$ ppm). This result provides the first direct evidence that intact RRV particles and isolated RRV VP8* interact with **5** in an identical mode and supports the theory that the initial virus–host cell contact is mediated by sialic acid binding to VP8*.

A number of X-ray crystal structures of **5** bound to RRV VP8* have been published.[5,18] Figure 3c shows the X-ray crystal structure of **5** bound to RRV VP8* protein.[5] The strong hydrophobic interaction of the the *N*-acetyl group with Tyr189 is evident. This interaction leads to a large STD NMR effect, whereas the solvent-exposed methyl aglycon results in no STD NMR signals.

We have also performed STD NMR competition experiments by adding **3** to a trypsin-activated RRV–**5** complex (Figure 3d,e). ^{1}H and ^{1}H STD NMR spectra were acquired (Figure 3h,i, respectively). The STD NMR spectrum of the RRV–**3**–**5** complex (Figure 3i) indicates that **3** does not completely displace the monosaccharide **5** from the binding sites on the RRV whole virus particles. Therefore **3**, presumably through the observed additional Gal unit interactions, marginally displaces **5** from the VP8* sialic acid binding site on the intact virions. The ^{1}H and ^{1}H STD NMR spectra of the RRV–**3** complex (Figure 3f,g, respectively) are shown for comparative purposes. This interpretation is consistent with a previous report[13] in which NMR-measured K_d values for **3** and **5** demonstrate that there is an enhanced affinity for **3** over **5**.

In summary, our results clearly demonstrate that while the sialic acid moiety of **3** is the primary recognition element of this ganglioside, the penultimate Gal residue does makes a contribution to the binding event. The reducing-end glucose moiety, perhaps not surprisingly, appears not to make contact with the protein. Furthermore, the bioactive conformation of the Sia–Gal fragment of **3**, as determined by NOE/trNOE spectroscopy and molecular modeling, is distinct from the

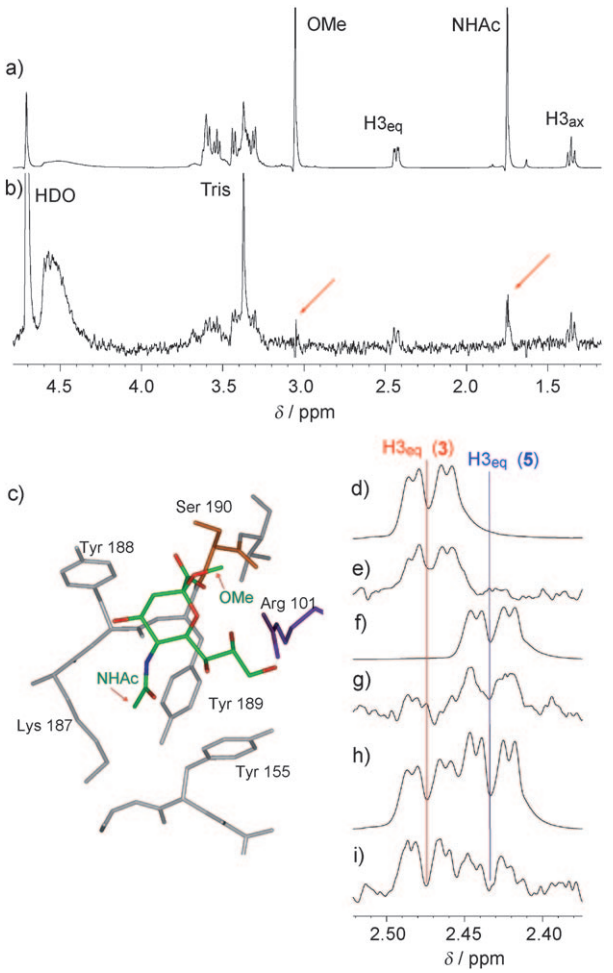

Figure 3. a) ^{1}H NMR and b) STD NMR spectra of **5** complexed with trypsin-activated RRV particles at 600 MHz and 288 K. c) X-ray crystal structure of **5** complexed with RRV VP8*[5] demonstrating the involvement of the *N*-acetamido group methyl protons, and the solvent-exposed methyl group of the aglycon moiety. d–i) Sections of the ^{1}H NMR and STD NMR spectra in the chemical-shift region of the H3$_{eq}$ protons: trypsin-activated RRV particles in complex with **3** (d and e), **5** (f and g) and a 0.8:1.0 mixture of **3** and **5** (h and i).

predominant solution conformation. From our STD NMR studies we hypothesize that the Sia–Gal fragment of the a-GM3 ganglioside mimics the terminal Sia–Gal portion of the GD1a-like receptors that we have recently determined to bind to the related rotavirus strain CRW-8.[2] Furthermore, the data presented here reveal that trypsin-activated RRV particles bind **3** in an identical mode to RRV virions not treated with trypsin. This finding clearly demonstrates that trypsin activation of rotavirus particles is not essential for glycan receptor recognition (adhesion). Therefore our results support the generally accepted model that rotavirus cell entry rather than cell attachment is activated by trypsin.[6,7,19] Finally, STD NMR studies of the methyl α-D-*N*-acetylneuraminide (**5**) bound to whole virus particles identify a virion binding epitope that is identical to that of the recombinantly expressed RRV VP8* protein.[3] This provides strong evidence that the initial rotavirus/host cell contact is substantially mediated by sialic acid binding to VP8*.

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