The GM2 Glycan Serves as a Functional Coreceptor for Serotype 1 Reovirus

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Abstract

Viral attachment to target cells is the first step in infection and also serves as a determinant of tropism. Like many viruses, mammalian reoviruses bind with low affinity to cell-surface carbohydrate receptors to initiate the infectious process. Reoviruses disseminate with serotype-specific tropism in the host, which may be explained by differential glycan utilization. Although α2,3-linked sialylated oligosaccharides serve as carbohydrate receptors for type 3 reoviruses, neither a specific glycan bound by any reovirus serotype nor the function of glycan binding in type 1 reovirus infection was known. We have identified the oligosaccharide portion of ganglioside GM2 (the GM2 glycan) as a receptor for the attachment protein σ1 of reovirus strain type 1 Lang (T1L) using glycan array screening. The interaction of T1L σ1 with GM2 in solution was confirmed using NMR spectroscopy. We established that GM2 glycan engagement is required for optimal infection of mouse embryonic fibroblasts (MEFs) by T1L. Preincubation with GM2 specifically inhibited type 1 but not type 3 reovirus infection of MEFs. To provide a structural basis for these observations, we defined the mode of receptor recognition by determining the crystal structure of T1L σ1 in complex with the GM2 glycan. GM2 binds in a shallow groove in the globular head domain of T1L σ1. Both terminal sugar moieties of the GM2 glycan, N-acetyneuraminic acid and N-acetylgalactosamine, form contacts with the protein, providing an explanation for the observed specificity for GM2. Reoviruses with mutations in the glycan-binding domain display diminished hemagglutination capacity, a property dependent on glycan binding, and reduced capacity to infect MEFs. Our results define a novel mode of virus-glycan engagement and provide a mechanistic explanation for the serotype-dependent differences in glycan utilization by reovirus.

Introduction

Virus infections are initiated by attachment of the virus to target cells of susceptible hosts. Receptors facilitate attachment, determine host range, and govern susceptibility of particular cells to infection. While viral attachment can be a monophasic event, this process frequently involves multiple receptors, and adhesion strengthening is a common mechanism that facilitates virus entry [1]. Thus, a virus may interact with an attachment factor, commonly a carbohydrate, to adhere via low-affinity interaction to the cell-surface, where it then binds to an additional receptor with high affinity that leads to viral entry. The identities of the low-affinity attachment factors are not known for many viruses.

Mammalian orthoreoviruses (reoviruses) serve as highly tractable models to study virus-receptor interactions. These viruses replicate to high titer, facilitating biochemical and biophysical studies, and both the virus and host can be manipulated genetically. Reoviruses contain ten segments of double-stranded RNA (dsRNA) encapsidated within two protein shells. Reoviruses can infect the gastrointestinal and respiratory tracts of a variety of mammals but rarely cause systemic disease outside of the immediate newborn period [2]. Most children are seropositive for reovirus by the age of 5 years [3]. Reoviruses preferentially infect tumor cells and are being tested in clinical trials for the treatment of a variety of cancers [4–6]. Reoviruses preferentially infect tumor cells more efficiently than untransformed cells, but it is likely that distribution, accessibility, and density of cellular receptors contribute to this process.

The three known reovirus serotypes are represented by the prototype strains type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D). These three strains differ markedly in cell tropism and viral spread, and these properties have been studied extensively using newborn mice [7]. T1L spreads hematogenously and infects ependymal cells, leading to non-lethal hydrocephalus.
Author Summary

Receptor utilization plays an important role in viral disease. Viruses must recognize a receptor or sometimes multiple receptors to infect a cell. Mammalian orthoreoviruses (reoviruses) serve as useful models for studies of viral receptor binding and pathogenesis. The reovirus experimental system allows manipulation of both the virus and the host to define mechanisms of viral attachment and disease. Like many viruses, reoviruses engage carbohydrate molecules on the cell-surface, but the oligosaccharide sequences bound and the function of glycans in binding infection were not known prior to this study. We used glycan screening to determine that serotype 1 reoviruses bind ganglioside GM2 and found that this interaction is required for efficient infection of some types of cells. To better understand how reovirus engages GM2, we determined the structure of the reovirus attachment protein σ1 in complex with the GM2 glycan and defined residues that are required for functional receptor binding. Reoviruses are being tested in clinical trials for efficacy in the treatment of cancer. Cancer cells commonly have altered glycan profiles. Therefore, understanding how reoviruses engage cell-surface glycans might lead to improvements in oncolytic therapy.

In this study, we employed glycan microarray analyses to identify ganglioside GM2 as a glycan receptor for reovirus T1L, and we used structural and infectivity data to define the glycan-protein interaction and the biological relevance of glycan binding to infection of host cells. Taken together, our structure-function data provide insight into how the GM2 glycan is specifically recognized by type 1 reoviruses and explain the serotype-specific nature of reovirus glycan utilization.

Receptor utilization plays an important role in viral disease. Viruses must recognize a receptor or sometimes multiple receptors to infect a cell. Mammalian orthoreoviruses (reoviruses) serve as useful models for studies of viral receptor binding and pathogenesis. The reovirus experimental system allows manipulation of both the virus and the host to define mechanisms of viral attachment and disease. Like many viruses, reoviruses engage carbohydrate molecules on the cell-surface, but the oligosaccharide sequences bound and the function of glycans in binding infection were not known prior to this study. We used glycan array screening to determine that serotype 1 reoviruses bind ganglioside GM2 and found that this interaction is required for efficient infection of some types of cells. To better understand how reovirus engages GM2, we determined the structure of the reovirus attachment protein σ1 in complex with the GM2 glycan and defined residues that are required for functional receptor binding. Reoviruses are being tested in clinical trials for efficacy in the treatment of cancer. Cancer cells commonly have altered glycan profiles. Therefore, understanding how reoviruses engage cell-surface glycans might lead to improvements in oncolytic therapy.
both L cells and MEFs are of murine origin, differences in sialic acid requirements are likely accounted for by differences in the expression on these cells of the known proteinaceous reovirus receptor, JAM-A. L cells, which do not require sialic acid for efficient entry, express higher levels of cell-surface JAM-A than do MEFs (Figure 1C). Thus, T1L may infect MEFs using an adhesion-strengthening mechanism in which binding to gangliosides must precede engagement of the relatively low abundance JAM-A receptor.

Glycan array screening identifies GM2 as a preferred ligand for T1L σ1

To assess the carbohydrate-binding specificity of T1L reovirus, we expressed and purified recombinant hexahistidine-tagged T1L σ1 protein for binding analyses in neoglycolipid-based glycan microarrays. Based on sequence alignment with T3D σ1, for which several crystal structures exist [18,24,25], two constructs were designed. The first construct, σ1long, comprised amino acids 261–470, which were predicted to fold into three ß-spiral repeats and the C-terminal head domain. The second construct, σ1short, comprised amino acids 300–470, which were predicted to form only the most C-terminal ß-spiral and the head domain. Both σ1 constructs included the predicted carbohydrate-binding site, which was reported to lie in close proximity to the head domain [27].

Glycan microarray analyses were carried out initially with σ1long using an array composed of 124 lipid-linked oligosaccharide probes. Among these are 119 sialylated probes with differing sialic acid linkages, backbone sequences, chain lengths, and branching patterns; five non-sialylated probes were included as negative controls (Table S1). The results from the glycan array screening showed a signal for the ganglioside GM2 that, despite its low intensity, was significantly stronger than the other signals (Figure S1). The GM2 glycan sequence contains two terminal sugars, Neu5Ac and N-acetylgalactosamine (GalNAc), that are both linked to a central galactose (Gal) via α2,3 and β1,4 linkages, respectively. The Gal is connected, via a β1,4 linkage, to a glucose (Glc), which is attached to a ceramide anchor.

Additional analyses were carried out with the σ1short construct, which was predicted to have less steric hindrance imposed by the long body domain and, therefore, to perhaps yield clearer results. Since the initial screen with σ1long revealed GM2 as a likely carbohydrate receptor, the second array was comprised of 21 ganglioside-related saccharide probes that included GM2 (Table S2). The results from this screen confirmed binding of the protein to GM2 and yielded a higher signal-to-noise ratio than the initial screen (Figure 2A). GM2 clearly exhibited the highest signal among the probes investigated, whereas several other structurally closely related probes (Figure 2B), e.g., the “a series” gangliosides GM3, GM1, and GD1a (sequences in Table S2), elicited marginally detectable low signals. The overall binding intensity of the σ1 protein, even with the short construct, is lower than that of other proteins tested in the same arrays, e.g., the VP1 proteins of polyoma viruses JCV and SV40, and the fiber knobs of adenovirus Ad37 (data not shown).

T1L σ1 interacts with the GM2 glycan in solution

To verify that T1L σ1 binds specifically to the GM2 glycan, we performed STD NMR spectroscopy experiments with σ1 and the glycan. This method is especially well suited to detect low-affinity binding between a large molecule, such as σ1, and a small oligosaccharide [36–38]. In an STD NMR experiment, the protein is selectively excited, and magnetization transfer to the ligand is observed if complex formation and rapid release of the ligand take place. If these conditions are fulfilled, the STD spectrum contains ligand resonances belonging to the binding epitope. A control experiment without protein serves to exclude direct excitation of the ligand. Using STD NMR, we found that T1L σ1 binds to the GM2 oligosaccharide in solution. Moreover, the STD analysis identified the protons of the carbohydrate that lie in close proximity (about 5 Å) to σ1 in the complex (Figure 2C, Figure S2A). All of the GM2 protons in the σ1-GM2 complex are part of the terminal Neu5Ac or the GalNAc moieties. The most prominent peak in the STD NMR spectrum belongs to the Neu5Ac methyl group, which receives considerably more saturation than the GalNAc methyl group. Protons H5, H6, H7, and one of the two H9 protons of Neu5Ac also are readily identified in the STD NMR spectrum, while the axial and equatorial H3 protons of this moiety receive little, if any, magnetization from the protein. Saturation transfer to the Neu5Ac protons H4 and H8 cannot be evaluated unambiguously because the resonances of both overlap with each other and with the GalNAc H6 resonance. Protons H1 through H4 of the GalNAc ring also are seen in the difference

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Figure 1. The effect of neuraminidase treatment on T1L infectivity in L cells and MEFs. (A) L cells or (B) MEFs were treated with A. ureafaciens neuraminidase for 1 h, followed by adsorption of T1L at an MOI of 10 or 100 PFU/cell, respectively. Cells were washed twice with PBS, and fresh medium was added. After incubation at 37°C for 20 h, cells were fixed, and reovirus antigen was detected by indirect immunofluorescence. Nuclei were stained with DAPI. The percentage of infected cells in three fields of view per well was determined. The results are expressed as the mean percent infected cells per well in triplicate wells for two independent experiments. Error bars represent standard deviations. (A) n.s., (B) ***, P<0.0001, as determined by two-tailed Student’s t test. (C) L cells or MEFs were stained with anti-JAM-A antibody followed by Alexa-488 labeled secondary antibody to measure cell-surface JAM-A expression. Fluorescence was detected by flow cytometry. Cells were gated on forward and side scatter and the mean fluorescence intensity (MFI) of Alexa-488 was quantified. Results shown are from a representative of three experiments each done with duplicate samples.

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spectrum, although they are generally less prominent than the Neu5Ac protons. No noteworthy transfer was observed for the GM2 galactose and glucose rings. Thus, the STD NMR spectroscopy data show that the T1L\textsubscript{1}-GM2 glycan interaction is based on contacts with ring atoms and the glycerol side chain of Neu5Ac, with additional contacts contributed by GalNAc ring atoms. The STD NMR experiment was repeated with the linear GM3 glycan (Figure S2B), which lacks the terminal GalNAc moieties.

Figure 2. T1L reovirus uses GM2 as a coreceptor. (A) Glycan microarray analysis of recombinant T1L\textsubscript{1} using 21 lipid-linked oligosaccharide probes. Each oligosaccharide probe was arrayed at four levels (as indicated) in duplicate. Numerical scores of the binding signals are means of duplicate spots (with error bars). The complete list of probes and their sequences are provided in Table S2. (B) Diagrams of “a series” gangliosides GM3, GM2, GM1, and GD1a present in the glycan array. Ceramide, glucose (Glc), galactose (Gal), N-5-acetyl neuraminic acid (Neu5Ac), and N-acetylgalactosamine (GalNAc) moieties are indicated. (C) STD NMR spectroscopy demonstrates that T1L\textsubscript{1} binds to the GM2 glycan in solution. Upper spectrum: $^1$H spectrum of the GM2 oligosaccharide alone; middle: STD spectrum of T1L\textsubscript{1} and the GM2 glycan; and lower spectrum: STD spectrum of the GM2 glycan alone as a control for direct excitation of the ligand. The protons are labeled and color-coded according to the sugar moieties within the GM2 oligosaccharide. The large peak just below 3.8 ppm unites the Neu5Ac H4 and H8 and the GalNAc H6 resonances.

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Figure 3. The effect of soluble glycans on T1L infectivity of MEFs. (A, B) T1L or (C) T3D (10⁷ PFU/well) were pre-incubated with the GM2 (A, C) or GM3 (B) glycan at the concentrations shown for 1 h prior to adsorption to MEFs at a final MOI of 100 PFU/cell. Cells were washed twice with PBS, and fresh medium was added. After incubation at 37°C for 20 h, cells were fixed and reovirus antigen was detected by indirect immunofluorescence. Nuclei were quantified by DAPI staining. The results are expressed as the mean percent infected cells per field in triplicate wells for two independent experiments. Error bars represent standard deviations. *, P < 0.05; **, P < 0.01; ***, P < 0.0001, as determined by two-tailed Student’s t test.

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Infection of MEF cells with T1L reovirus is blocked by preincubation with the GM2 glycan

To investigate whether GM2 serves as a functional receptor for T1L reovirus, we tested the soluble GM2 glycan for the capacity to inhibit T1L infection of MEFs. Preincubation of the GM2 glycan with T1L resulted in a dose-dependent decrease in T1L infectivity (Figure 3A). However, preincubation of T1L with the GM3 glycan diminished infectivity to a lesser extent and was not dose-dependent (Figure 3B). As a specificity control, incubation of reovirus T3D with the GM2 glycan did not diminish the capacity of T3D to infect MEFs (Figure 3C). These findings demonstrate that the GM2 glycan is specifically recognized by T1L and serves as a physiologically relevant coreceptor.

Crystal structure of T1L σ1 in complex with the GM2 glycan

To visualize interactions between T1L σ1 and its coreceptor, we determined the crystal structure of the σ1 long construct in complex with the GM2 glycan. The overall structure of the monomer and the organization of the trimer are similar to the T3D σ1 structure [10]. The crystalized T1L σ1 protein folds into three β-spiral repeats and a globular C-terminal head domain (Figure 4A–C). The head domain, comprising amino acids 327–470, is constructed from two Greek-key β-strands (β-strands A–D and E–H), β-spiral repeats 1 (amino acids 310–326) and 3 (residues 268 to 287) form motifs, each consisting of four α-helical turns, with both prolines being in the cis-configuration, β-spiral repeats 2 (amino acids 288–305) and 3 (residues 268 to 287) form motifs, each consisting of four α-helical turns, with both prolines being in the cis-configuration. The Neu5Ac residue contributes the majority of the contacts between GM2 and T1L σ1 and is wedged into a shallow groove bordered on each side by β-strands B and C. Additional contacts involve the GalNAC moiety. The lactose component, which forms the backbone of the branched glycan and would be linked to the ceramide anchor in the GM2 ganglioside, points away from the protein. The mobilities of the sugar moieties are reflected in their thermal factors (B-factors). The average B-factors of Neu5Ac and GalNAC are in the same range as those of the neighboring protein residues, indicating nearly complete occupancy of the glycan-binding pockets (Table 1). The remaining two sugars, and especially the glucose moiety, have elevated B-factors, in agreement with their lack of contacts to protein residues and resultant higher mobility (Table 1).

The Neu5Ac residue can be unambiguously placed in the electron density map due to unique identifying features of this sugar compound (Figure 5A, B). The N-acetyl and glycerol chains of Neu5Ac insert between β-strands B and C, where they form hydrogen bonds with backbone atoms of both β-strands (Figure 5A, B). Additionally, the methyl group of the Neu5Ac N-acetyl chain inserts into a hydrophobic pocket flanked by V354, F369, and M372, consistent with the dominance of this group in the STD NMR spectrum. The side chain of Q371 likely forms a hydrogen bond with the Neu5Ac carboxylate. However, at 3.6 Å resolution, the conformations of protein side chains cannot be unambiguously determined.

There are two possible orientations for the GalNAc group as a result of the electron density map. For our crystallographic model, we selected the sugar conformation that is favored according to the

present on GM2. The difference spectrum demonstrates that the GM3 trisaccharide interacts with T1L σ1 and that saturation transfer is observed to Neu5Ac protons only. The STD NMR experiments allow no direct estimate of relative affinities for GM2 and GM3, but it is likely that T1L σ1 binds with greater affinity to the GM2 glycan because of the additional contacts with the terminal GalNAc of this compound. This assumption is consistent with our observation that the GM2 binding signal on the microglycan array is much higher compared with the GM3 signal (Figure 2A).
corresponding Carbohydrate Ramachandran plot (CaRp) (Figure S3, Table S3) [40]. This orientation of GalNAc also is preferred by GM2 in solution as assessed by NMR spectroscopy [41]. The GalNAc moiety does not form any hydrogen bonds with T1L, but it clearly interacts with the protein through van der Waals contacts (Figure 5A). Similar contacts are made for each of the two possible orientations of the GalNAc ring.

Crystal structure of T1L σ1 in complex with the GM3 glycan

The GM3 glycan differs from the GM2 oligosaccharide in lacking the GalNAc moiety (Figure 2B). Although GM3 exhibited only very weak binding to T1L σ1 in the glycan arrays (Figure 2A), the structure of T1L σ1 in complex with the GM2 glycan indicated that GM3 contains most of the essential features for complex formation and could potentially engage T1L σ1, albeit with lower affinity compared to GM2. We therefore determined a crystal structure of T1L σ1 in complex with the GM3 glycan at 3.5 Å resolution (Table 2). The structure shows that T1L σ1 binds to the GM3 glycan at the same site as the GM2 glycan, using identical contacts for the Neu5Ac group (Figure 6). The Neu5Ac residues of the T1L σ1-GM3 and T1L σ1-GM2 complex structures superimpose with an r.m.s.d. value of 0.76 Å (Figure S4). As is the case for the T1L σ1-GM2 complex, the lactose moiety of the GM3 glycan points away from the protein.

Residues in T1L reovirus required for carbohydrate engagement

To identify residues in T1L σ1 required for glycan binding, we generated T1L reoviruses carrying point mutations in the GM2-
Table 1. Data collection and refinement statistics for the T1L σ1-GM2 complex.

| Data collection          |                  |
|--------------------------|------------------|
| Resolution (Å)           | 50.3-60 (3.69-3.60) |
| Space group              | P321             |
| a, c (Å)                 | 147.5, 164.5     |
| α, β, γ (°)              | 90, 90, 120      |
| Rmerge (%)               | 11.5 (61.9)      |
| CC1/2 (%)<sup>*</sup>    | 99.8 (88.1)      |
| λ(Å)                     | 1.0              |
| ϕ/ψ(°)                   | 17.2 (3.1)       |
| Completeness (%)         | 99.9 (99.8)      |
| Redundancy               | 6.2              |

| Refinement               |                  |
|--------------------------|------------------|
| Rwork/Rfree (%)**        | 18.5/20.4        |
| B-factors                |                  |
| Chain A (Å<sup>2</sup>)  | 86.6             |
| Chain B (Å<sup>2</sup>)  | 86.9             |
| Chain C (Å<sup>2</sup>)  | 98.7             |
| GM2-A (complete) (Å<sup>2</sup>) | 99.4 |
| Neu5Ac/GalNAc-A (Å<sup>2</sup>) | 85.9/89.0 |
| GM2-B (complete) (Å<sup>2</sup>) | 101.2 |
| Neu5Ac/GalNAc-B (Å<sup>2</sup>) | 87.9/94.3 |
| GM2-C (complete) (Å<sup>2</sup>) | 111.4 |
| Neu5Ac/GalNAc-C (Å<sup>2</sup>) | 95.2/108.3 |
| Number of atoms          |                  |
| Protein                  | 4776             |
| GM2 glycan               | 171              |
| r.m.s.d.                 |                  |
| Bond lengths (Å)         | 0.01             |
| Bond angles (°)          | 1.11             |
| Ramachandran Plot        |                  |
| Favored (%)              | 593 (97.5)       |
| Allowed (%)              | 15 (2.5)         |
| Outliers (%)             | 0                |

<sup>r.m.s.d. = root-mean-square deviation.</sup>
<sup>*CC<sub>1/2</sub> = correlation coefficient ([90]).</sup>
<sup>**R<sub>work</sub>/R<sub>free</sub> was calculated with 10% of the data.</sup>

We observed that each of these residues is in close proximity to the bound glycan (Figure 5B). For point mutants V354F, V354L, and M372L, the amino acids present in T1L σ1 were replaced with residues predicted to partially block the putative Neu5Ac-binding pocket. Residue Q371 was replaced with an acidic residue to introduce a negative charge that was expected to repel the Neu5Ac moiety and interfere with binding to the GM2 glycan (Figure 5B). Point mutants S370P, Q371A, and M372F were generated to replace a T1L σ1 residue with the corresponding residue in T3D σ1, which does not bind a carbohydrate receptor via its head domain [19] (Figure 5C). The S1 genes of all mutant viruses were sequenced to confirm the fidelity of mutagenesis.

We thought it possible that mutations within the putative carbohydrate-binding site might result in diminished infectivity in MEFs due to impaired glycan engagement or some other impairment in viral fitness. To eliminate the latter possibility and normalize infectious units for the virus strains tested, we used L cells, which do not require sialylated glycan engagement to support infection, likely due to an abundance of JAM-A on the cell surface. Unlike our findings with MEFs, neither neuraminidase treatment of cells (Figure 1) nor pretreatment of virus with GM2 (data not shown) altered T1L infectivity in L cells. To determine whether the mutant σ1 proteins are properly folded, we tested the conformation-sensitive monoclonal antibody (mAb) 5C6 for the capacity to inhibit mutant virus infection of L cells. Neutralization-resistant T1L mutants selected by mAb 5C6 have alterations at Q417 and G447 in T1L σ1 [43]. These residues are located at the upper part of the T1L σ1 head domain, close to the intersubunit interface (Figure 7A). An antibody that recognizes these residues likely binds a trimeric conformer of the T1L σ1 head and thus indicates the presence of properly folded and assembled σ1 trimers. Preincubation with mAb 5C6 significantly diminished the capacity of wildtype and mutant T1L viruses to infect L cells (Figure 7B), suggesting that the σ1 head domain of the mutants is recognized by mAb 5C6 and not grossly misfolded.

To test whether the σ1 point mutants have impaired glycan binding, we quantified the capacity of wildtype and mutant viruses to agglutinate human erythrocytes (Figure 8), a property linked to carbohydrate binding [20]. All of the mutants had a significant defect in hemagglutination, with alterations of V354, S370, and Q371 showing the greatest impairment. To determine whether the point mutants have an altered capacity to infect cells in a carbohydrate-dependent fashion, we quantified infectivity in MEFs, which require carbohydrate binding for optimal infection (Figure 1). MEFs were inoculated with wildtype and mutant viruses at an MOI of 1 FFU/cell for each virus as equilibrated in assays using L cells. The V354F, S370P, Q371A, and Q371E mutants displayed a significant defect in infectivity in MEFs (Figure 9). Taken together, these data suggest that residues V354, S370, and Q371, which flank the carbohydrate-binding site of T1L σ1, are required for functional engagement of the GM2 glycan.

**Discussion**

Although all known reovirus serotypes utilize JAM-A as a receptor, they display striking differences in viral tropism and spread. These differences segregate with the S1 gene, which encodes the σ1 attachment protein [7]. The σ1 residues that interact with JAM-A are conserved among the serotypes [25], and serotype-dependent tropism in the CNS is observed in JAM-A-null mice [11]. These observations suggest that serotype-dependent differences in host disease are attributable to σ1 engagement of cell-surface receptors other than JAM-A.

T3D σ1 binds to sialic acid using residues in its body domain, interacting with α2,3, α2,6, and α2,8-linked sialic acid in a similar manner [19,27]. Although hemagglutination data [20] and lectin-based studies [30] demonstrate that T1L interacts with α2,3-linked sialic acid, neither the identity of the specific glycan nor the molecular basis of T1L-glycan interactions was known. In this study, we found that T1L uses the GM2 glycan as a functional receptor, which is the first identification of a specific glycan recognized by any reovirus serotype.

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The GM2 Glycan Is a Receptor for Type 1 Reovirus
Hemagglutination assays have been used in many previous studies of reovirus-glycan interactions [27–29]. Reovirus displays serotype-dependent hemagglutination profiles. Type 1 reoviruses agglutinate human but not bovine erythrocytes, whereas type 3 reoviruses preferentially agglutinate bovine erythrocytes and agglutinate human erythrocytes less efficiently [29]. These observations suggest that the glycan-binding sites of type 1 and type 3 reovirus are distinct, a hypothesis that is now confirmed by this study and that of Reiter, et al [19]. Analysis of the respective crystal structures sheds light on the potential species differences in hemagglutination behavior. Whereas human erythrocytes express the Neu5Ac form of sialic acid [44], bovine cells express mostly Neu5Gc and less Neu5Ac [45]. The additional hydroxyl group of Neu5Gc would face a hydrophobic pocket in the type 1 s-binding site, making a favorable interaction unlikely. In contrast, the type 3 s-binding site likely could accommodate either Neu5Ac or Neu5Gc (D.M. Reiter and T. Stehle, unpublished data).

The GM2 glycan binds to the head domain of T1L s and not, as predicted earlier, to the body region of the protein [27]. It is possible that cell-surface structures in addition to glycans contribute to hemagglutination by type 1 reovirus and this may explain why the chimeric s proteins used in the earlier study had diminished, but not abolished, hemagglutination capacity. Alternatively, disruption of the neck domain of s in the chimeric proteins used in the previous study [27] might have altered the conformation of the glycan-binding domain in the head.

Inspection of the carbohydrate-binding site reveals that the two terminal sugar moieties of the branched GM2 glycan, Neu5Ac and GalNAc, contact T1L s, Neu5Ac and less efficiently [29]. These observations suggest that the glycan-binding sites of type 1 and type 3 reovirus are distinct, a hypothesis that is now confirmed by this study and that of Reiter, et al [19]. Analysis of the respective crystal structures sheds light on the potential species differences in hemagglutination behavior. Whereas human erythrocytes express the Neu5Ac form of sialic acid [44], bovine cells express mostly Neu5Gc and less Neu5Ac [45]. The additional hydroxyl group of Neu5Gc would face a hydrophobic pocket in the type 1 s glycan-binding site, making a favorable interaction unlikely. In contrast, the type 3 s-binding site likely could accommodate either Neu5Ac or Neu5Gc (D.M. Reiter and T. Stehle, unpublished data).

Although the GM3 oligosaccharide is also able to bind T1L s in solution, infectivity studies indicate that GM2 is the preferred glycan receptor for T1L reovirus. While preincubation with either GM2 or GM3 oligosaccharides resulted in diminished infectivity of MEFs, the GM2 glycan blocked infectivity more efficiently and in a dose-dependent fashion. The “extra” GalNAc moiety of GM2 is likely responsible for the selectivity of T1L s for this glycan. At only 41 Å², the surface area in T1L s buried by interactions with GalNAc is very small compared to the 284 Å² surface buried by contacts with Neu5Ac in the same complex (Table S4), but the small additional interactions are nevertheless expected to mediate higher-affinity binding of the GM2 glycan compared with GM3, which lacks GalNAc. In addition, due to its branched structure, the GM2 glycan has less conformational freedom in solution than the linear GM3 molecule [41], which may also facilitate interactions with the virus. Entropy furthermore favors binding

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**Figure 5. The carbohydrate-binding site of T1L s.** (A) Surface representation of T1L s shown in light gray. The GM2 glycan is depicted in stick representation with the two terminal sugars, Neu5Ac and GalNAc, that contact T1L s shown in color, and the Gal and Glc residues shown in gray. (B) Close-up view of the Neu5Ac-binding pocket, with contacting residues shown in stick representation in blue (carbons) and the protein surface shown in light gray. Neu5Ac is depicted in stick representation and colored as in Figure 4. Hydrogen bonds between T1L s and Neu5Ac are represented with black dashes. The methyl group of the N-acetyl chain of Neu5Ac inserts into a hydrophobic pocket formed by residues V354, F369, and M372. (C) Sequence alignment of the carbohydrate-binding site of T1L s (amino acids 350–380) with the corresponding region of T3D s (residues 333–363). The two β-strands forming the carbohydrate-binding site of T1L s are highlighted in blue. The four residues included in the mutational analyses are marked with blue dots.

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of the branched GM2 glycan over the linear GM3 molecule. In support of this idea, limited conformational freedom of the branched glycan GM1 is essential for its selective engagement by cholera toxin over related compounds [46]. Therefore, the branched sequence of the GM2 glycan sequence is preferred over cholera toxin over related compounds [46]. Therefore, the support of this idea, limited conformational freedom of the branched GM2 glycan over the linear GM3 molecule. In altering V354, S370, and Q371 having the greatest effect displayed impaired hemagglutination capacity, with mutations introducing mutations into the glycan-binding site. All mutants identify residues required for functional glycan engagement by T1L coreceptor. Type 3 reoviruses differing only in the capacity to engage cell-surface glycans display marked differences in tropism for future research. The precise tissue distribution of GM2 is not completely understood, but the glycan is a component of the mammalian nervous system [50–52]. In mice, T1L reovirus infects ependymal cells and causes hydrocephalus [8,53]. The presence of GM2 in the brain provides an attractive explanation for the use of this coreceptor by T1L. Because ganglioside expression may differ in cell types that serve as targets for reovirus infection in vivo, there may be cells in which one glycan or another predominates as a T1L coreceptor. Type 3 reoviruses differing only in the capacity to engage cell-surface glycans display marked differences in tropism [54,55]. We anticipate that glycan binding also functions in the pathogenesis of type 1 reovirus infections, which is an area of current investigation in our laboratories.

Reovirus is being tested in clinical trials as an oncolytic adjunct to conventional cancer therapy. Some tumor cells have altered ganglioside expression compared with untransformed cells, and some overexpress GM2 [56–58]. Humanized antibodies directed against GM2 prevent the formation of organ metastases in mice with small-cell lung cancer [59]. It is possible that ganglioside overexpression in

---

**Table 2.** Data collection and refinement statistics for the T1L σ1-GM3 complex.

| Data collection |   |   |
|-----------------|---|---|
| Resolution (Å)  | 50.3-50 (3.59-3.50) |   |
| Space group     | P3221 |   |
| a, c (Å)        | 149.4, 165.2 |   |
| NeutAc-A (Å²)   | 91.4 |   |
| NeutAc-B (Å²)   | 81.3 |   |
| NeutAc-C (Å²)   | 91.4 |   |
| GM3-A (complete) (Å²) | 104.1 |   |
| GM3-B (complete) (Å²) | 101.4 |   |
| Total reflections | 107527 (8217) |   |
| Unique reflections | 26751 (1984) |   |
| Rwork/Rfree (%)** | 18.6/19.7 |   |
| B-factors       |   |   |
| Chain A (Å²)    | 81.4 |   |
| Chain B (Å²)    | 83.4 |   |
| Chain C (Å²)    | 90.5 |   |
| Number of atoms | 4794 |   |
| Protein         | 118 |   |
| r.m.s.d.        |   |   |
| Bond lengths (Å) | 1.09 |   |
| Bond angles (°) | 0.01 |   |
| Ramachandran plot |   |   |
| Favored (%)     | 602 (98.9) |   |
| Allowed (%)     | 6 (1.0) |   |
| Outliers (%)    | 1 (0.2) |   |

*CC1/2 = correlation coefficient ([90]).

r.m.s.d. = root-mean-square deviation.

Rwork/Rfree (%)** = calculated with 10% of the data.

**Table 2.** Data collection and refinement statistics for the T1L σ1-GM3 complex.

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The GM2 Glycan Is a Receptor for Type 1 Reovirus

The precise tissue distribution of GM2 is not completely understood, but the glycan is a component of the mammalian nervous system [50–52]. In mice, T1L reovirus infects ependymal cells and causes hydrocephalus [8,53]. The presence of GM2 in the brain provides an attractive explanation for the use of this coreceptor by T1L. Because ganglioside expression may differ in cell types that serve as targets for reovirus infection in vivo, there may be cells in which one glycan or another predominates as a T1L coreceptor. Type 3 reoviruses differing only in the capacity to engage cell-surface glycans display marked differences in tropism [54,55]. We anticipate that glycan binding also functions in the pathogenesis of type 1 reovirus infections, which is an area of current investigation in our laboratories.

Reovirus is being tested in clinical trials as an oncolytic adjunct to conventional cancer therapy. Some tumor cells have altered ganglioside expression compared with untransformed cells, and some overexpress GM2 [56–58]. Humanized antibodies directed against GM2 prevent the formation of organ metastases in mice with small-cell lung cancer [59]. It is possible that ganglioside overexpression in
tumor cells alters the susceptibility of certain cancers to reovirus infection. Understanding the molecular basis of reovirus-glycan interactions might improve the design of effective oncolytics.

Although T1L and T3D reoviruses bind sialylated glycans as receptors using their \( \sigma_1 \) proteins, the locations of the respective carbohydrate-binding sites differ substantially (Figure 1A, B). The T1L \( \sigma_1 \) glycan-binding site resides in the head domain. In contrast, the T3D \( \sigma_1 \) glycan-binding site is in the N-terminal part of the body domain, close to the midpoint of the \( \sigma_1 \) molecule. Structure and sequence comparisons show that the head of T3D \( \sigma_1 \) would not be capable of engaging Neu5Ac-based receptors because the carbohydrate-binding site of the T1L \( \sigma_1 \) head is blocked in T3D \( \sigma_1 \) (Figures 5C, 10). It also is unlikely that the region of T1L \( \sigma_1 \) corresponding to the T3D \( \sigma_1 \) glycan-binding site would interact with sialic acid. T3D \( \sigma_1 \) residue Arg202 forms critical interactions with Neu5Ac and, in T1L \( \sigma_1 \), there is an aspartate instead of an arginine at the equivalent position. The negatively charged aspartate side chain would probably repel Neu5Ac and, thus, carbohydrate engagement at this site is impeded (Figure 11C). The different locations of the carbohydrate-binding sites contrast with the conserved interactions of both \( \sigma_1 \) proteins with JAM-A. The JAM-A-binding sites of both T1L and T3D \( \sigma_1 \) proteins are located at the base of the head domain, and interactions between \( \sigma_1 \) and JAM-A are similar in both serotypes [25,26]. Assuming that both protein- and carbohydrate-binding sites are accessible for both serotype 1 and serotype 3 reoviruses, it is possible that the mechanisms of attachment are not conserved between the reovirus serotypes, which may contribute to the observed differences in viral tropism and spread.

**Materials and Methods**

**T1L \( \sigma_1 \) protein expression and purification**

Construct \( \sigma_1_{long} \) comprises the three most C-terminal predicted \( \beta \)-spiral of T1L \( \sigma_1 \) and the head domain (amino acids 261–470). Construct \( \sigma_1_{short} \) comprises the most C-terminal predicted \( \beta \)-spiral of T1L \( \sigma_1 \) and the head domain (amino acids 300–470). Expression and purification of T1L \( \sigma_1_{long} \) and T1L \( \sigma_1_{short} \) were facilitated by attaching a trimeric version of the GCN4 leucine zipper [60,61] to the N-terminus of the \( \sigma_1 \) sequence, similar to the strategy we used to express T3D \( \sigma_1 \) [19]. The \( \sigma_1 \) construct was cloned into the pQE-80L expression vector (Qiagen), which includes a non-cleavable N-terminal His\(_6\)-tag. The protein was expressed in *E. coli* Rosetta 2 (DE3) (Novagen) by autoinduction at 20°C for 48 to 72 h. Bacteria were lysed using an EmulsiFlex (Avestin) homogenizer and purified via Ni-affinity chromatography (His-Trap FF column, GE Healthcare). The fusion protein was eluted from the column, and the protein solution was desalted using a PD10 desalting column (GE Healthcare). The GCN4 domain and the His\(_6\)-tag were removed from the fusion protein using 1 \( \mu \)g trypsin per mg protein at 20°C for 4 h. The resultant products were subjected to size-exclusion chromatography (Superdex 200) to remove the tags, trypsin, and other minor impurities.
Undigested versions of both constructs were used for glycan array screening. STD NMR experiments were performed using $\sigma_1$long. Both constructs were used for structural analysis. Uncleaved $\sigma_1$short yielded crystals diffracting to 2.6 Å resolution. This higher resolution structure was used as a reference model for refinement of the lower-resolution structures of cleaved $\sigma_1$long in complex with the GM2 or GM3 glycan.

Glycan microarray analyses

Microarrays were composed of lipid-linked oligosaccharide probes, neoglycolipids (NGLs) and glycolipids, robotically printed on nitrocellulose-coated glass slides at 2 and 7 fmol per spot using a non-contact instrument, and analyses were performed as described [62,63]. For analysis of T1L $\sigma_1$long, the results of 124 oligosaccharide probes (5 non-sialylated and 119 sialylated, Glycosciences Array Set 40–41), at 5 fmol per spot are shown in Figure S1 and Table S1. For the analysis of T1L $\sigma_1$short, a different version of the microarray (in house designation Ganglioside Dose Response Array set 1) was used; results of the 21 ganglioside-related probes (Table S2) each arrayed at four levels: 0.3, 0.8, 1.7 and 5.0 fmol/spot, are shown in Figure 2A.

For the initial analysis of His-tagged T1L $\sigma_1$long, the protein was incubated with mouse monoclonal anti-poly-histidine (Ab1) and biotinylated anti-mouse IgG antibodies (Ab2) (both antibodies...
Figure 9. Infectivity of σ1 mutant viruses in MEFs. Monolayers of MEFs were adsorbed with the strains shown at an MOI of 1 FFU/field (as titered in L cells) at room temperature for 1 h. Cells were washed twice with PBS, and fresh medium was added. After incubation at 37°C for 20 h, cells were fixed, and reovirus antigen was detected by indirect immunofluorescence. Nuclei were stained with DAPI. The percentage of infected cells in three fields of view per well was determined. The results are from a representative experiment of three experiments performed with triplicate wells. Error bars represent standard deviations. **, P<0.01, as determined by two-tailed Student’s t test.
doi:10.1371/journal.ppat.1003078.g009

Figure 10. The head domain of T3D σ1 does not bind Neu5Ac. (A) Surface representation of T1L σ1 depicted in gray. (B) SSM superposition of T1L (gray) and T3D (cyan) σ1. The GM2 glycan is shown in stick representation (colors as in Figure 4) in both panels. Clashes between the carbohydrate and T3D σ1 are highlighted with red circles in panel B. Both the Neu5Ac and GalNAc moieties of the GM2 oligosaccharide would clash with T3D σ1 residues.
doi:10.1371/journal.ppat.1003078.g010
Figure 11. Comparison of the receptor-binding sites of T1L and T3D σ1. Surface representations of (A) T3D σ1 in complex with the GM3 glycan (PDB accession code 3S6X) and (B) T1L σ1 in complex with the GM2 glycan. The carbohydrates are shown in stick representation and colored as in Figure 4. The JAM-A-binding sites are highlighted in green, and the carbohydrate-binding sites in T1L and T3D are depicted in pink and blue, respectively. (C) Sequence alignment of the carbohydrate-binding site in T1L and T3D σ1. Residues required for carbohydrate engagement in T3D σ1 are highlighted in blue. Residue R202, which forms a central interaction with Neu5Ac in T3D σ1, is marked with a blue dot.

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from Sigma) at a ratio of 4:2:1 (by weight). The σ1long-antibody complexes were prepared by preincubating Ab1 with Ab2 at ambient temperature for 15 min, followed by addition of Histagged T1L σ1long and incubation on ice for 15 min. The σ1long-antibody complexes were diluted in 5 mM HEPES (pH 7.4), 150 mM NaCl, 0.3% (v/v) Blocker Casein (Pierce), 0.3% (v/v) bovine serum albumin (Sigma), 5 mM CaCl2 and 40 mM imidazole (referred to as HBS-Casein/BSA-imidazole), to provide a final σ1long concentration of 150 μg/ml, and overlaid onto the arrays at 20 °C for 2 h. Binding was detected using Alexa Fluor 647-labeled streptavidin.

Crystallization, x-ray structure determination, and refinement

Crystals of uncleaved σ1short formed in 0.1 M MES/imidazole (pH 6.5), 10% PEG 4000, 20% glycerol, 0.02 M sodium formate, 0.02 M ammonium acetate, 0.02 M trisodium citrate, 0.02 M sodium potassium L-tartrate, 0.02 M sodium oxamate at 4°C using the sitting-drop-vapor-diffusion method. No additional cryoprotection was necessary. Crystals of σ1long formed in 0.1 M Na cacodylate (pH 6.0–6.6), 1.2–1.5 M (NH4)2SO4 at 4°C using the sitting-drop-vapor-diffusion method. For preparation of complexes, these crystals were transferred to 20 mM GM2 or GM3 oligosaccharide (Elicityl) in the crystallization solution for 5–10 min. Prior to flash-freezing, the crystals were transferred to a solution containing 0.1 M Na cacodylate, 1.34 M (NH4)2SO4, 25% glycerol, and 20 mM GM2 or GM3 glycan.

The crystals belonged to space group P3_21 and contained one trimer in the asymmetric unit. A complete data set was collected at the Swiss Light Source, beamline X06SA. XDS was used to index and scale the reflection data [65]. The structure was determined by molecular replacement with Phaser (CCP4) [66],[67] using the coordinates of T1L σ1 derived from the previously determined T1L σ1-JAM-A complex structure as a search model [26]. Manual model building was carried out using coot [68]. Structural refinement was performed using Refmac5 (CCP4) [69], Phenix [70], and autoBUSTER [71],[72].

Inspection of the 2Fo-Fc maps for the structures of the T1L σ1-glycan complexes revealed clear, unambiguous electron density for most of the GM2 and GM3 oligosaccharides at a 1.5 σ contour level. The glycans also were visible in difference electron density maps. The unbiased electron density maps in Figures 4, 6, and S3 show the initial Fo-Fc maps of the T1L σ1-GM2 and T1L σ1-GM3 glycan complexes obtained after molecular replacement using the previously solved structure of unliganded T1L σ1. The carbohydrates were included in the model at this point. Refinement of the ligands was performed using the CCP4 library and user-defined constraints. Structure images were created using PyMOL [73]. Coordinates and structure factors of both complexes have been deposited in the Protein Data Bank with accession codes 4GU3 (T1L-σ1-GM2 glycan complex) and 4GU4 (T1L σ1-GM3 glycan complex).

Sequence and structural analysis

Sequence alignments were performed using T-Coffee [74] and analyzed using Jalview [75,76]. Structure alignments were calculated by secondary-structure matching (SSM) superposition in coot [77]. The Ramachandran plot was generated with Rampage (CCP4) [78]. Buried surface areas were calculated using AreaImol (CCP4) [79,80].

STD NMR spectroscopy

NMR spectra were recorded using 3 mm tubes and a Bruker AVIII-600 spectrometer equipped with a room temperature probe head at 283 K and processed with TOPSPIN 3.0 (Bruker). Samples containing 1 mM GM2 or GM3 glycan (Elicityl), 20 mM potassium phosphate (pH 7.4), and 150 mM NaCl with and without 20 μM T1L σ1 were used for the STD NMR measurements and the frequency control, respectively. Samples were prepared in D2O, and no additional water suppression was used to preserve the anemonic proton signals. The sample without
protein also was used for spectral assignment. The off- and on-resonance irradiation frequencies were set to −50 ppm and 7.3 ppm, respectively. The irradiation power of the selective pulses was 57 Hz, the saturation time was 2 s, and the total relaxation delay was 3 s. A 50 ms continuous-wave spin-lock pulse with a strength of 3.2 kH was employed to suppress residual protein signals. A total number of 312 scans were recorded. A total of 10,000 points were collected, and spectra were multiplied with a Gaussian window function prior to Fourier transformation. Spectra were referenced using HDO as an internal standard [81].

Cells

Spinnaker-adapted murine L cells were grown in suspension culture in Joklik’s minimum essential medium (Lonza) supplemented to contain 5% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 25 ng/ml amphotericin B (Sigma-Aldrich). MEFs were generated from C57/Bl6 mice at embryonic day 13.5 as described [82]. MEFs were maintained in Dulbecco’s modified Eagle’s minimum essential medium (DMEM) (Gibco) supplemented to contain 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1X MEM nonessential amino acids (Sigma-Aldrich), 20 mM HEPES, and 0.1 mM 2-mercaptopetanol (Sigma-Aldrich). Cells at passages 3–6 were used in this study.

Viruses and plasmid-based reovirus rescue

Viruses were generated using plasmid-based reverse genetics [42,83]. BHK-T7 cells (5×105) were seeded in 60 mm tissue-culture dishes (Corning) and allowed to incubate at 37°C overnight. OptiMEM (Invitrogen) (0.75 ml) was mixed with 55.25 μl TransIT-LT1 transfection reagent (Mirus) and incubated at RT for 20 min. Plasmid constructs representing cloned gene segments from the T1L genome, pT7S1 T1L, pT7S2 T1L, pT1L3S3 T1L, pT7S4 T1L, pT17M1 T1L, pT17L1M2 T1L, and pT17L2M3 T1L were mixed into the OptiMEM/TransIT-LT solution. Equal amounts of each plasmid were added for a total of 17.75 μg DNA. The plasmid-transfection solution was added to BHK-T7 cells and incubated for 3–5 days. Following two freeze-thaw cycles, recombinant viruses were isolated by plaque purification using L-cell monolayers [84]. Purified virions were generated using second-passage L cell-lysat stocks. Viral particles were Freon-extracted from infected cell lysates and layered onto 1.2 to 1.4 g/cm² CaCl gradients and centrifuged at 62,000 xg for 18 h. Bands were collected and dialyzed exhaustively in virion-storage buffer as described [12,85]. To generate mutant viruses, residues V334, S370, Q371, and M372 in the S1 gene plasmid were altered by QuickChange (Stratagene) site-directed mutagenesis. S1 resides V354, S370, Q371, and M372 in the S1 gene plasmid were altered by QuickChange (Stratagene) site-directed mutagenesis. Viruses were generated using plasmid-based reverse genetics [42,83]. Purified reovirus virions (1011 particles) were distributed into 96-well U-bottom microtiter plates (Costar) and allowed to incubate at 37°C overnight. OptiMEM (Invitrogen) (0.75 ml) was mixed with 55.25 μl TransIT-LT1 transfection reagent (Mirus) and incubated at RT for 20 min. Plasmid constructs representing cloned gene segments from the T1L genome, pT7S1 T1L, pT7S2 T1L, pT1L3S3 T1L, pT7S4 T1L, pT17M1 T1L, pT17L1M2 T1L, and pT17L2M3 T1L were mixed into the OptiMEM/TransIT-LT solution. Equal amounts of each plasmid were added for a total of 17.75 μg DNA. The plasmid-transfection solution was added to BHK-T7 cells and incubated for 3–5 days. Following two freeze-thaw cycles, recombinant viruses were isolated by plaque purification using L-cell monolayers [84]. Purified virions were generated using second-passage L cell-lysat stocks. Viral particles were Freon-extracted from infected cell lysates and layered onto 1.2 to 1.4 g/cm² CaCl gradients and centrifuged at 62,000 xg for 18 h. Bands were collected and dialyzed exhaustively in virion-storage buffer as described [12,85]. To generate mutant viruses, residues V334, S370, Q371, and M372 in the S1 gene plasmid were altered by QuickChange (Stratagene) site-directed mutagenesis. S1 resides V354, S370, Q371, and M372 in the S1 gene plasmid were altered by QuickChange (Stratagene) site-directed mutagenesis.

Flow cytometry

To determine the relative amount of JAM-A on L cells and MEFS, 5×10⁵ cells were incubated with rat anti-mouse JAM-A at a dilution of 1:200 followed by staining with Alexa-488 labeled goat anti-rat IgG at 1:1000. All staining was done in PBS supplemented to contain 5% FBS. Fluorescence was measured using an LSRII BD, Vanderbilt University Flow Cytometry Shared Resource). Mean fluorescence intensity of a forward and side scatter gated population was determined using FlowJo software.

Hemagglutination assay

Purified reovirus virions (10¹¹ particles) were distributed into 96-well U-bottom microtiter plates (Costar) and serially diluted twofold in 0.05 ml of PBS. Human type O erythrocytes (Vanderbilt Blood Bank) were washed twice with PBS and resuspended at a concentration of 1% (vol/vol). Erythrocytes (0.05 ml) were added to wells containing virus particles and incubated at 4°C for 3 h. A partial or complete shield of erythrocytes on the well bottom was interpreted as a positive HA result; a smooth, round button of erythrocytes was interpreted as a negative result. HA titer is expressed as 10¹¹ particles divided by the number of particles/HA unit. One HA unit equals the number of particles sufficient to produce HA.

Statistical analysis

Statistical analysis was performed using Prism (Graphpad). Two-tailed Student’s t tests were used for all infectivity studies.
The hemagglutination assays were analyzed using a one-way Anova followed by a Bonferroni’s correction. P values of less than 0.05 were considered to be statistically significant.

Supporting Information

Figure S1 Glycan microarray analyses of T1L-σ1long using a microarray of 124 lipid-linked oligosaccharide probes. Numerical scores of the binding signals are means of duplicate spots at 5 fmol/spot (with error bars). The various types of terminal sialic acid linkage are indicated by the colored panels as defined at the bottom of the figure. Error bars are all relatively large due to the low fluorescent signals. The list of probes and their sequences and binding scores are provided in Table S1. The X indicates an artifact on the slide giving a false signal resulting in a large error bar.

(TIF)

Figure S2 STD NMR spectroscopy of T1L σ1 with GM2 and GM3 oligosaccharide. (A) Chemical structure of the GM2 glycan. Protons that receive saturation upon binding to T1L σ1 are color-coded according to the corresponding STD NMR spectrum in Figure 2C. (B) T1L σ1 binds to the GM3 glycan in solution. STD NMR experiment of T1L σ1 and the GM3 oligosaccharide. Upper spectrum: 1H spectrum of the GM3 glycan solution. STD NMR experiment of T1L at 3.0 s PLOS Pathogens | www.plospathogens.org 15 December 2012 | Volume 8 | Issue 12 | e1003078

(A) T1L σ1 and the GM3 glycan; middle: STD spectrum of T1L σ1 and the GM3 glycan; and lower spectrum: STD spectrum of the GM3 oligosaccharide alone to ensure that no direct excitation of the glycan takes place. A schematic drawing of GM3 is provided in the upper left corner.

(TIF)

Figure S3 CaRp analysis of the T1L σ1-GM2 complex. CaRp analysis (Carbohydrate Ramachandran plot, www.glycosciences.de) of the three GM2 oligosaccharide molecules in the T1L σ1-GM2 complex. A schematic of the GM2 oligosaccharide is included with the three glycosidic bonds numbered. The structure of one GM2 glycan molecule and its unbiased F,-F, map at 3.0 σ contour level for 2.0 Å are shown at the bottom right.

(TIF)

Figure S4 T1L σ1 binds Neu5Ac of the GM2 glycan and the GM3 glycan at the same site. SSN superposition of the T1L σ1-GM2 complex (yellow) and the T1L σ1-GM3 complex (cyan). The protein chains are shown as ribbon tracings, and the Neu5Ac moieties of the GM2 and GM3 glycan are depicted in stick representation in yellow and cyan, respectively. They superimpose with an r.m.s.d. value of 0.76 Å.

(TIF)

Table S1 Oligosaccharide probes used in the initial glycan microarray analyses, sorted by sialyl linkage and backbone sequence, and the binding signals (means of the fluorescence intensity at ~5 fmol/probe spot) of T1L-σ1long.

(DOC)

Table S2 List of probes and sequences included in the ganglioside dose-response array set.

(DOC)

Table S3 Dihedral angles of the glycosidic linkages of the three GM2 oligosaccharides bound to T1L σ1.

(DOC)

Table S4 T1L σ1 surface areas buried by GM2 and GM3 in the σ1-glycan complex structures.

(DOC)

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Author Contributions

Conceived and designed the experiments: KR JES YL TSD TS. Performed the experiments: KR JES YL BSB. Analyzed the data: KR JES YL BSB DMR TF TSD TS. Wrote the paper: KR JES YL BSB TF TSD TS.

References

1. Haywood AM (1994) Virus receptors: binding, adhesion strengthening, and changes in viral structure. J Virol 68: 1–5.
2. Drozdov TS, Parker J, Sherry B (2012) Orthoreovirus. In: D.M. K, P.M. H. editors. Fields Virology. 6th edition. Philadelphia: Lippincott Williams & Wilkins.
3. Tai JH, Williams J, Edwards KM, Wright PF, Crowe JE, et al. (2005) Prevalence of reovirus-specific antibodies in young children in Nashville, Tennessee. J Infect Dis 191: 1221–1224.
4. Coffey MC, Strong JE, Forsyth PA, Lee PW (1998) Reovirus therapy of tumors using a microarray of 124 lipid-linked oligosaccharide probes. PLoS Pathog 7: e1002166.
5. Weiner HL, Powers ML, Fields BN (1980) Absolute linkage of virulence and reovirus virulence: role of the S1 gene segment. Science 233: 770–774.
6. Lupas A, Van Dyke M, Stock J (1991) Predicting coiled coils from protein sequences. Science 252: 1162–1164.
7. Duncan R, Horne D, Cashdollar LW, Jolik JK, Lee PW (1990) Identification of conserved domains in the cell attachment proteins of the three serotypes of reovirus. Virology 174: 399–409.
8. Weiner HL, Powers ML, Fields BN (1980) Absolute linkage of virulence and central nervous system cell tropism of reoviruses to viral hemagglutinin. J Infect Dis 141: 699–616.
9. Morrision LA, Sielman RM, Fields BN (1991) Direct spread of reovirus from the intestinal lumen to the central nervous system through vagal autonomic nerve fibers. Proc Natl Acad Sci U S A 88: 3852–3856.
10. Antar AA, Konopka JL, Campbell JA, Henry RA, Per bidgozo AL, et al. (2009) Functional adhesion molecule-A is required for hematogenous dissemination of reovirus. Cell Host Microbe 5: 59–71.
11. Reiter DM, Frierson JM, Halvorson EE, Kobayashi T, Dermody TS (2011) The reovirus sigma1 protein is a determinant of hematogenous but not neural virus dissemination in mice. J Virol 85: 11781–11790.
12. Lee PW, Hayes EC, Jolik JK (1981) Protein sigma 1 is the reovirus cell attachment protein. Virology 108: 136–163.
13. Lee PW, Hayes EC, Jolik JK (1981) Protein sigma 1 is the reovirus cell attachment protein. Virology 108: 136–163.
14. Furlong DR,olibert ML, Fields BN (1988) Sigma 1 protein of mammalian reoviruses extends from the surfaces of viral particles. J Virol 62: 246–256.
15.olibert ML, Dermody TS, Fields BN (1990) Structure of the reovirus cell attachment protein: a model for the domain organization of sigma 1. J Virol 64: 2976–2989.
16. Weiner HL, Powers ML, Fields BN (1980) Absolute linkage of virulence and central nervous system cell tropism of reoviruses to viral hemagglutinin. J Infect Dis 141: 699–616.
17. Duncan R, Horne D, Cashdollar LW, Jolik JK, Lee PW (1990) Identification of conserved domains in the cell attachment proteins of the three serotypes of reovirus. Virology 174: 399–409.
18. Wilkins. The staff in the Vanderbilt University Flow Cytometry Shared Resource for technical support.

Author Contributions

Conceived and designed the experiments: KR JES YL TSD TS. Performed the experiments: KR JES YL BSB. Analyzed the data: KR JES YL BSB DMR TF TSD TS. Wrote the paper: KR JES YL BSB TF TSD TS.
21. Campbell JA, Schelling P, Wetzel JD, Johnson EM, Forrest JC, et al. (2005) Junctional adhesion molecule a serves as a receptor for prototype and field-isolate strains of mammalian reovirus. J Virol 79: 7967–7978.

22. Prota AE, Campbell JA, Schelling P, Forrest JC, Watson MJ, et al. (2006) Crystal structure of human junctional adhesion molecule-1: implications for reovirus binding. Protac Nat Acad Sci U S A 100: 5366–5371.

23. Liu Y, Nusrat A, Schnell FJ, Reaves TA, Walsh S, et al. (2000) Human junctional adhesion molecule regulates tight junction remodeling in epithelia. J Cell Sci 113 (Pt 13): 2393–2474.

24. Schilling P, Guglielmi KM, Kirchner E, Partzold B, Demory TS, et al. (2007) The reovirion sigma1 acidastic sandwich: a trimerization mode poised for conformational change. J Biol Chem 282: 11502–11509.

25. Kirchner E, Guglielmi KM, Straus HM, Demory TS, Stehle T (2008) Structure of reovirus sigma1 in complex with its receptor junctional adhesion molecule-A. PLoS Pathog 4: e1000235.

26. Kirchner E (2009) Structural and functional studies of the reovirus attachment protein sigma1 and its interaction with the receptor JAM-A. Elektronische Ressource. pp. Online-Ressource.

27. Chappell JD, Duong JL, Wright BW, Demory TS (2000) Identification of carbohydrate-binding domains in the attachment proteins of type 1 and 3 reoviruses. J Virol 74: 8722–8727.

28. Lerner AM, Cherry JD, Finland M (1963) Hemagglutination with reoviruses. Virology 19: 36–65.

29. Gomatos PJ, Tamm I (1962) Reactive sites of reovirus type 3 and their interaction with receptor substance. Virology 15: 435–461.

30. Helander A, Silvey KJ, Mantis NJ, Hutchings AB, Chandraan K, et al. (2003) The viral sigma1 protein and glycoconjugates containing alpha2-6-linked sialic acid are involved in type 1 reovirus adherence to M cell apical surfaces. J Virol 77: 7769–7777.

31. Paul RW, Lee PW (1967) Glycophorin is the reovirus receptor on human erythrocytes. Virology 55: 194–91.

32. Nibert ML, Chappell JD, Dermody TS (1995) Infectious subvirion particles of reovirus type 1 Dearing exhibit a loss in infectivity and contain a cleaved sigma1 protein. J Virol 69: 3057–3067.

33. Barton ES, Connolly JL, Forrest JC, Chappell JD, Dermody TS (2001) Utilization of sialic acid as a coreceptor enhances reovirus attachment by multistep receptor-mediated endocytosis. J Virol 76: 2200–2211.

34. Shevchuk NA, Hathout Y, Epifano O, Su Y, Liu Y, et al. (2007) Alteration of ganglioside synthesis by GM3 synthase knockout in murine embryonic fibroblasts. Biochem Biophys Acta 1717: 1226–1234.

35. Yossef-Nagar, Fajuanji R, Kaseling R, Welsh RM (1982) Interferon-induced alterations in sialic acid and glycoconjugates of L-929 cells. Virology 121: 363–371.

36. Mayer M, Meyer B (1999) Characterization of ligand binding by saturation transfer difference NMR spectroscopy. Angewandte Chemie International Edition 38: 1784–1788.

37. Meyer B, Peters T (2003) NMR Spectroscopy techniques for screening and identifying ligand binding to protein receptors. Angewandte Chemie-International Edition 42: 864–890.

38. Neu U, Hengel H, Blaum BS, Maciej et al. (2012) Structures of Merck cell polyomavirus VP1 complexes define a sialic acid binding site required for infection. PLoS Pathog 8: e1002738.

39. van Kaaij MJ, van der Meij H, Lavesque G, Coopmans MP (1989) A beta-spiral in the adenovirus fiber shaft reveals a new structural motif for a fibrous protein. Nature 401: 935–938.

40. Lutteke T, Frank M, von der Lieth CW (2005) Carbohydrate Structure Guide (CSS): analysis of carbohydrate 3D structures derived from the PDB. Nucleic Acids Res 33: D242–246.

41. Lavery SB (1991) IH-NMR study of GM2 ganglioside: evidence that an interresidue amida-carboxyl hydrogen bond contributes to stabilization of a preferred conformation. Glycoconj J 8: 484–492.

42. Kobayashi T, Ooms LS, Bicker M, Chappell JD, Demory TS (2010) An improved reverse genetics system for mammalian orthoreoviruses. Virology 390: 194–200.

43. Hender lot A, Miller CL, Myers KS, Neutra MR, Nibert ML (2004) Protective immunoglobulin A and G antibodies bind to overlapping intersubunit epitopes in the head domain of type 1 reovirus adhesin sigma1. J Virol 78: 10695–10705.

44. Muchmore EA, Diaz S, Varia K (1998) A structural difference between the cell surface of humans and the great apes. J Mol Phys Anthrop 107: 107–198.

45. Musielak M (2004) Are there two functionally distinguished Neu5Gc pools with different functions? Biochim Biophys Acta 1676: 196–208.

46. Watanabe T, Pikel CS, Takeyama H, Lloyd KO, Shiku H, et al. (1992) Human melanoma antigen MH is an autoantigenic ganglioside related to GD2. J Exp Med 165: 1804–1809.

47. Cahan LD, Irie RF, Singh R, Cassardini A, Paulson JG (1982) Identification of a human neuroneodermal tumour antigen (OFA-I-2) as ganglioside GD2. Proc Nat Acad Sci U S A 79: 7629–7633.

48. Yamada T, Bandoh H, Takeuchi L, Kata K, Li Q, et al. (2011) Genetically engineered humanized anti-ganglioside GM2 antibody against multiple organ metastasis was produced by GM2-expressing small-cell lung cancer cells. Cancer Sci 102: 2157–2163.

49. Harbury PB, Zhang T, Kim PS, Alber T (1993) A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. Science 260: 1407–1413.

50. Harbury PB, Kim PS, Alber T (1994) Crystal structure of an leucine zipper trimmer. Nature 371: 80–83.

51. Palma AS, Feizi T, Zhang Y, Stoll MS, Lawson AM, et al. (2006) Ligands for the beta-galactosialidase receptor, Dectin-1, defined using "designer" microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides. J Biol Chem 281: 5771–5779.

52. Palma AS, Zhang Y, Chirli RL, Campanerio-Rhodes MA, Liu Y, et al. (2012) Neoglycolipid-based "designer" microarrays to define beta-galactosidic ligands for Dectin-1. Methods Mol Biol 808: 337–359.

53. Stoll M, Feizi T. Software Tools for Storing, Processing and Displaying Carbohydrate Microarray Data. In: Ketterm C, editor. 4–5 October, 2009. Potsdam, Germany. Frankfurt, Germany: Belaite Institute for the Advance-ment of Chemical Sciences. pp. 123–140.

54. Baksh W (2010) Xds. Acta Crystallogr D Biol Crystallogr 66: 125–132.

55. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, et al. (2007) Phaser crystallographic software. J Appl Crystallogr 40: 231–237.

56. Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCoy AJ, et al. (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr D Biol Crystallogr 50: 59–77.

57. Yamada T, Bandoh H, Takeuchi L, Kata K, Li Q, et al. (2011) Genetically engineered humanized anti-ganglioside GM2 antibody against multiple organ metastasis was produced by GM2-expressing small-cell lung cancer cells. Cancer Sci 102: 2157–2163.

58. Smart OS, Womack T, Flensburg C, Keller P, Paciorek W, et al. (2012) Human neuroectodermal tumor antigen (OFA-I-2) as ganglioside GD2. Proc Nat Acad Sci U S A 79: 7629–7633.
81. Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, et al. (1995) 1H, 13C and 15N chemical shift referencing in biomolecular NMR. J Biomol NMR 6: 135–140.
82. Danthi P, Prajierss AJ, Berger AK, Holm GH, Zinkel SS, et al. (2010) Bid regulates the pathogenesis of neurotropic reovirus. PLoS Pathog 6: e1000980.
83. Kobayashi T, Antar AA, Boehme KW, Danthi P, Eby EA, et al. (2007) A plasmid-based reverse genetics system for animal double-stranded RNA viruses. Cell Host Microbe 1: 147–157.
84. Virgin HWt, Bassel-Duby R, Fields BN, Tyler KL (1988) Antibody protects against lethal infection with the neurally spreading reovirus type 3 (Dearing). J Virol 62: 4594–4604.
85. Smith RE, Zweerink HJ, Jeldik WK (1969) Polypeptide components of virions, top component and cores of reovirus type 3. Virology 39: 791–810.
86. Wilson GJ, Wetzel JD, Puryear W, Bassel-Duby R, Dermody TS (1996) Persistent reovirus infections of L cells select mutations in viral attachment protein sigma1 that alter oligomer stability. J Virol 70: 6598–6606.
87. Wetzel JD, Chappell JD, Pogo AE, Dermody TS (1997) Efficiency of viral entry determines the capacity of murine erythroleukemia cells to support persistent infections by mammalian reoviruses. J Virol 71: 299–306.
88. Virgin HWt, Mann MA, Fields BN, Tyler KL (1991) Monoclonal antibodies to reovirus reveal structure/function relationships between capsid proteins and genetics of susceptibility to antibody action. J Virol 65: 6772–6781.
89. Iskarapatyoti JA, Willis JZ, Guan J, Ashley Morse E, Ikizler M, et al. (2012) A rapid, automated approach for quantitation of rotavirus and reovirus infectivity. J Virol Methods 184: 1–7.
90. Karpas PA, Diederichs K (2012) Linking crystallographic model and data quality. Science 336: 1030–1033.