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The VP8* Domain of Neonatal Rotavirus Strain G10P[11] Binds to Type II Precursor Glycans

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Naturally occurring bovine-human reassortant rotaviruses with a P[11] VP4 genotype exhibit a tropism for neonates. Interaction of the VP8* domain of the spike protein VP4 with sialic acid was thought to be the key mediator for rotavirus infectivity. However, recent studies have indicated a role for nonsialylated glycoconjugates, including histo-blood group antigens (HBGAs), in the infectivity of human rotaviruses. We sought to determine if the bovine rotavirus-derived VP8* of a reassortant neonatal G10P[11] virus interacts with hitherto uncharacterized glycans. In an array screen of >600 glycans, VP8* P[11] showed specific binding to glycans with the Galβ1-4GlcNAc motif, which forms the core structure of type II glycans and is the precursor of H type II HBGA. The specificity of glycan binding was confirmed through hemagglutination assays; GST-VP8* P[11] hemagglutinates type O, A, and B red blood cells as well as pooled umbilical cord blood erythrocytes. Further, G10P[11] infectivity was significantly enhanced by the expression of H type II HBGA in CHO cells. The bovine-origin VP4 was confirmed to be essential for this increased infectivity, using laboratory-derived reassortant viruses generated from sialic acid binding rotavirus SA11-4F and a bovine G10P[11] rotavirus, B223. The binding to a core glycan unit has not been reported for any rotavirus VP4. Core glycan synthesis is constitutive in most cell types, and modification of these glycans is thought to be developmentally regulated. These studies provide the first molecular basis for understanding neonatal rotavirus infections, indicating that glycan modification during neonatal development may mediate the age-restricted infectivity of neonatal viruses.

Interaction with cell surface glycans is a critical step in the initiation of many enteric infections (1). For rotavirus, this is mediated by the outer capsid spike protein VP4 through its glycan binding domain, VP8*. Sialic acid (Sia) is a key binding partner for VP8*; previous studies have shown that some animal rotaviruses bind to terminal Sia residues on cell surfaces and some human rotaviruses interact with gangliosides, such as GM1, that contain internal Sia moieties (2–6). Recently, some human rotaviruses have been shown to interact with nonsialylated glycoconjugates, including histo-blood group antigens (HBGAs) that are found in mucosal secretions and on the surface of epithelial cells (7–9). Structural studies on the VP8* of one such human rotavirus, HAL1166, showed that HBGA binding occurs in the same pocket where Sia binds in animal rotaviruses and that binding is mediated by specific sequence changes in this region. Further, the infectivity of HAL1166 is enhanced by HBGA expression *in vitro* (7). These new data are shifting the paradigm in understanding human rotavirus infectivity, and while they may explain zoonotic transmission of some animal rotaviruses, their relevance to different human strains remains unclear.

Human rotaviruses are widely known to be the leading cause of diarrheal mortality in children worldwide. However, rotavirus infections in neonates are distinct from those in older children. Neonatal infections are predominantly asymptomatic and are often associated with unusual rotavirus strains. These strains appear to be geographically restricted, show remarkable stability, and can persist in specific settings for long periods of time (10, 11). Rotaviruses are classified into G and P genotypes using a binary nomenclature system based on the sequence variability in the genes encoding the outer capsid glycoprotein VP7 (G type) and protease-sensitive spike protein VP4 (P type) (12). A group of unusual rotaviruses possessing the P[11] VP4 type and a G9 or G10 VP7 genotype has been associated with neonatal infections in India. Of these, the G10P[11] rotaviruses were initially detected in diarrheal samples from cattle in many studies and in some asymptomatic infections in neonates (13–15). The understanding of the epidemiology of these viruses, however, changed when a 4-year hospital surveillance study involving 1,300 neonates in southern India showed that nearly 50% of neonates were positive for rotavirus and 81% of the samples were genotyped as G10P[11]; furthermore, the virus was significantly associated with gastrointestinal symptoms in this study (16). In concomitant hospital-based surveillance studies for rotavirus diarrhea among 960 infants from >1 month to 36 months of age, only 1 case of G10P[11] was seen out of 342 rotavirus-positive cases, clearly indicating a predilection of this strain for neonates (17). A high incidence of neonatal infections with the G10P[11] strain was also detected in a community-based birth cohort in this region, confirming the predilection of this strain for neonates (18, 19). Through whole-ge-

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nome sequencing of a clinical isolate, the neonatal G10P[11] virus was identified to be a bovine-human reassortant, with the genes encoding nonstructural proteins NSP1 to NSP3 being of human rotavirus origin and the remaining 8 genes, including VP4, being of bovine rotavirus origin (20). Another P[11] rotavirus strain associated with neonatal infections is G9P[11]. This strain causes asymptomatic neonatal infections in northern India and forms the basis for a new rotavirus vaccine currently in phase III trials (21, 22). G9P[11] is also a bovine-human reassortant virus, with all genes being of human rotavirus origin except VP4, which is of bovine rotavirus origin (23, 24).

Given the high disease burden of rotavirus gastroenteritis and the cocirculation of many genotypes, including globally prevalent strains, the predominance of a single genotype in this age-restricted population sharply contrasts with the strain diversity seen among older children (16, 17, 25). With recent evidence for the interaction of the VP8* of human rotaviruses with novel glycan partners, we sought to determine if the bovine rotavirus-derived VP8* of neonatal G10P[11] rotavirus interacts with hitherto uncharacterized glycan partners and whether this interaction is significant for infection.

MATERIALS AND METHODS

Comparison of VP8* sequences. To determine if the VP8* amino acid sequence of P[11] rotaviruses was different from that of other strains with known glycan partners, multiple alignments were performed using the Clustal W algorithm on BioEdit software (version 7.0.5.3). The G10P[11] sequences included those from (i) a human neonatal isolate (N155) whose whole genome has previously been sequenced (20), (ii) a human neonatal isolate (N1509) that has been adapted to culture through multiple passages on African green monkey kidney epithelial (MA104) cells, and (iii) a bovine G10P[11] isolate (strain B223). Both N155 and N1509 were isolated from symptomatic neonates with feed intolerance. The VP8* amino acid sequences of P[11] rotaviruses were compared to the VP8* amino acid sequences of other animal and human rotaviruses with known glycan partners (Fig. 1). These included sialidase-sensitive animal rotavirus strains that bind to glycans with a terminal Sia moiety (rhesus rotaviruses RRV and SA11, porcine rotavirus CRW-8) and human rotaviruses that bind to glycans with internal Sia residues (Wa and DS-1). Wa and DS-1 represent the two most common global genotypes, G1P[8] and G2P[4], respectively. In addition, the VP8* amino acid sequence of a P[14] sialidase-insensitive human rotavirus, HAL1166, that binds to A-type HBGA at the same location as Sia in the VP8* of animal rotaviruses was also included in the analysis.

GST-VP8* protein expression and purification. The gene sequences of VP8* corresponding to amino acids (aa) 64 to 224 of human neonatal G10P[11] rotaviruses N155 and N1506 as well as the bovine G10P[11] rotavirus B223 were synthesized (Epoch Life Science) and cloned with an N-terminal glutathione *S*-transferase (GST) tag into a pGEX-2T expression vector (GE Healthcare) (7). The recombinant proteins were expressed in *Escherichia coli* BL21(DE3) cells (Novagen) and purified using glutathione Sepharose 4 Fast Flow chromatography medium (GE Healthcare). The VP8* segments of SA11 variant 4F (SA11-4F) and human rotavirus Wa were also expressed and purified for use as controls. The purified GST-VP8* proteins were used in glycan array and hemagglutination assays.

Glycan array screen for GST-VP8* proteins. The carbohydrate binding specificity of GST-VP8* P[11] was determined using a glycan array comprised of >600 glycans (v5.0; Consortium for Functional Glycomics, Protein-Glycan Interaction Core-H, Emory University School of Medicine [http://www.functionalglycomics]) as previously described (7). Briefly, the recombinant protein from neonatal G10P[11] N155 was used in decreasing concentrations (200, 20, 2.0, and 0.2 μg/ml) in individual arrays and detected using a fluorescently labeled anti-GST monoclonal antibody (Sigma). The strength of binding to a glycan was expressed in terms of relative fluorescence units (RFU). To normalize the results between the different arrays, a rank was assigned to each glycan using the formula 100 × (test glycan RFU/highest number of RFU in that assay). An average of the ranks determined the final rank for each glycan (26). Similar glycan array studies were carried out with GST-VP8* proteins from SA11-4F, Wa, N1509, and B223. These proteins were tested in a single concentration (200 μg/ml) on the array.

Hemagglutination assay. Adult human red blood cells (RBCs) corresponding to blood types O, A1, A2, and B were obtained from Immucor Inc. Pooled umbilical cord blood erythrocytes were obtained from Mybiosource. The cells were centrifuged for 10 min at 500 × *g*, and 0.5% suspensions of each RBC type were prepared in 0.85% saline (pH 6.2). The GST-VP8* proteins were serially diluted on 96-well V-bottom plates (Nunc) and mixed with an equal volume of each RBC suspension. The suspension was incubated for about 1 h at 4°C and assessed for hemagglutination. The hemagglutination titer was recorded as the highest dilution of sample that resulted in complete hemagglutination. Recombinant Norwalk virus virus-like particles (VLPs) known to hemagglutinate type A and type O but not type B RBCs and GST-VP8* P[14] from rotavirus strain HAL1166, which hemagglutinates type A RBCs, were included as positive controls. The assay was also performed with GST to rule out false positivity due to the GST tag.

To determine the effect of neuraminidase treatment of RBCs on hemagglutination, O-type RBCs were treated with neuraminidase from *Vibrio cholerae* (Sigma). Briefly, a 10% suspension was prepared using 10 μl of packed RBCs in 250 mM sodium acetate buffer (pH 5.8) containing bovine serum albumin (1.25 mg/ml), NaCl (5 mM), and $CaCl_2$ (4 mM). The RBCs were treated with or without 25 mU neuraminidase for 2 h at 37°C (modified from reference 27). Following incubation, the cells were washed and hemagglutination assays were carried out as described above. The neuraminidase treatment studies were carried out with a subset of the GST-VP8* proteins. These included VP8* from human and bovine G10P[11] rotaviruses (N155 and B223, respectively), SA11-4F, and HAL1166.

Cell lines and virus strains for binding and infectivity assays. Binding and infectivity assays were performed on Chinese hamster ovary (CHO) cells that differ in the production of HBGAs (7). The parental CHO cells do not express HBGAs (H−/A−/B−). Single-transfectant CHO cells that stably express the enzyme fucosyltransferase (Fut2) express the H antigen (H+/A−/B−). Double-transfectant CHO cells expressing Fut2 and either A-type glycosyltransferase or B-type glycosyltransferase express the A antigen (H+/A+/B−) or B antigen (H+/A−/B+), respectively (28). Polylactosamines were assayed on CHO cells by flow cytometry using the biotinylated lectins STL from *Solanum tuberculosum* (potato; Biovalley), LEL from *Lycospersum* (tomato; Biovalley), and streptavidin-peridinin chlorophyll protein conjugate (BD Biosciences) at 1 μg/ml.

The binding studies were carried out using VP8* P[11] from N155 and VP8* P[14]. Infectivity on CHO cells was assessed using a panel of virus strains. These included the human neonatal G10P[11] rotavirus isolate (N1509) that was adapted to culture through multiple passages on MA104 cells, well-characterized laboratory strains (simian rotavirus SA11 variant 4F [SA11-4F], human rotaviruses Wa and HAL1166, and bovine G10P[11] rotavirus B223), as well as 6 reassortant viruses (R-179, R-144, R-141, R-491, R-004, and R-198) generated using SA11-4F and B223 as parental strains. The reassortant viruses differed in their overall genetic background (see Fig. 6), and their characterization has been described previously (29, 30).

Binding and infectivity assays on CHO cells. Binding of VP8* to the different CHO cells was assessed by flow cytometry using fluorescein isothiocyanate (FITC)-labeled GST-VP8* proteins. The binding studies were carried out on parental CHO cells or CHO cells stably expressing the H-type or the A-type glycosyltransferase with FITC-GST-VP8* P[11] from human neonatal G10P[11] N155. FITC-GST and FITC-GST-V8*

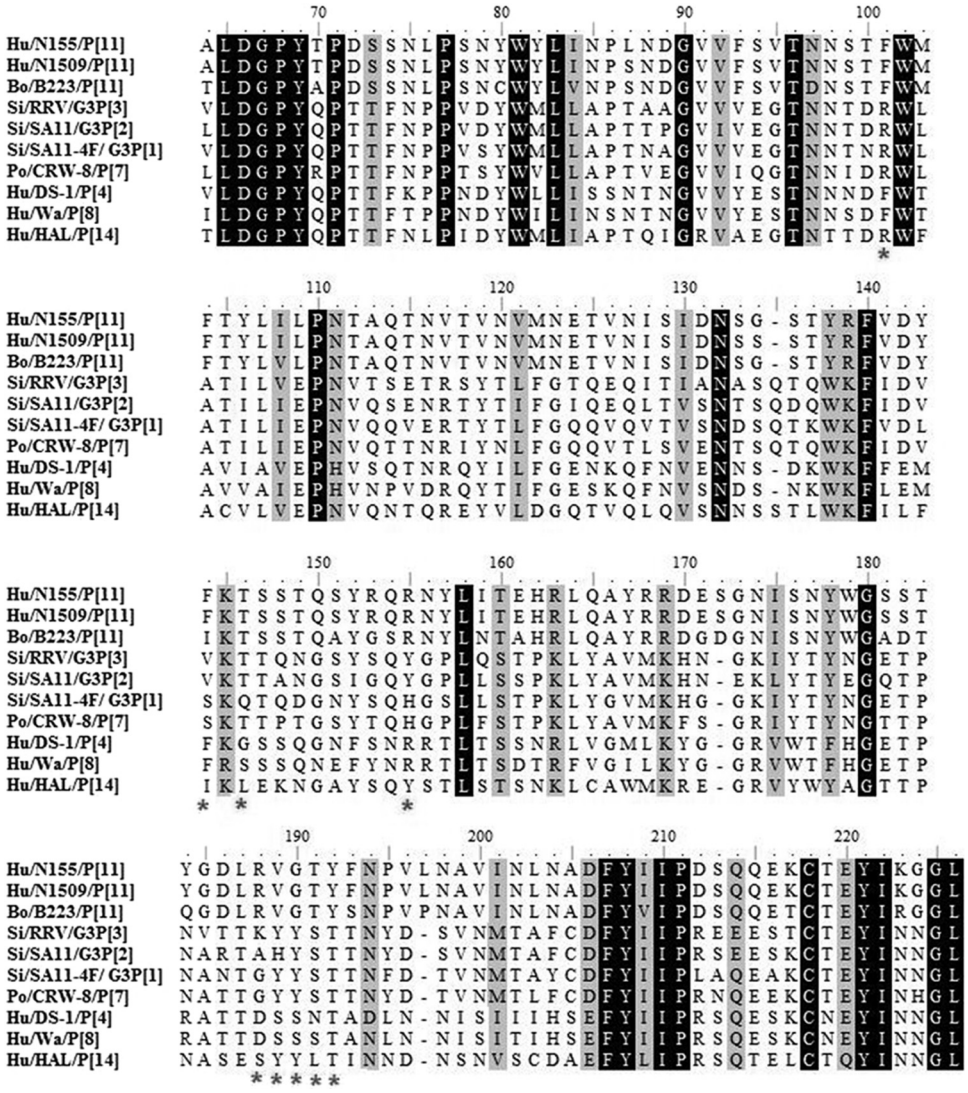


FIG 1 The VP8* P[11] sequence is distinct from the sequence in rotavirus strains with known glycan partners. Alignment of the VP8* amino acid sequences from neonatal G10P[11] rotavirus isolates (N155 [GI:164632843] and culture-adapted neonatal isolate N1509) with the VP8* amino acid sequences from a bovine (Bo) G10P[11] isolate (B223; GI:408959), simian (Si) rotaviruses RRV (GI:61869) and SA11 (GI:61892), SA11 variant 4F (GI:61946), porcine (Po) rotavirus CRW-8 (GI:226699783), and human (Hu) rotavirus strains Wa (GI:333781), DS-1 (GI:28268531), and HAL1166 (GI: 452131) is shown. The species of origin, the names of the isolates, and their VP4 types are indicated. *, residues known to interact with Sia for the animal viruses RRV, SA11, and CRW-8. The VP8* amino acid sequence of cell culture-adapted human neonatal rotavirus strain N1509 G10P[11] was >98% identical to that of clinical isolate N155. N155 and N1509 showed the same amino acid changes in the Sia or HBGA binding pocket as RRV, CRW-8, and HAL1166.

P[14] from HAL1166 were included as controls. Briefly, 2 mg/ml of purified recombinant GST, GST-VP8* P[11], or P[14] in phosphate-buffered saline (pH 8.0) was labeled using an FITC antibody-labeling kit (Thermo Scientific). The proteins were incubated with one vial of FITC reagent each at room temperature for 1 h with protection from light. The labeled proteins were then purified using the purification resin provided in the kit and stored at 4°C before examining their binding to CHO cells. The binding studies were carried out at 4°C for 30 min using 25 μg of protein for 1×10^6 to 2×10^6 cells. In addition, binding studies were also carried out following treatment of CHO cells with neuraminidase at 37°C for 1 h at a concentration of 50 mU per 10^6 cells.

Virus titers on the parental and the singly and doubly transfected CHO cell lines were determined using fluorescent focus assays (3). Briefly, the cells were grown to confluence on 96-well plates (Costar; Corning) and inoculated with 2-fold dilutions of trypsin-activated virus. After 12 to 14 h of infection, the cells were fixed with ice-cold methanol and stained with a rabbit polyclonal antirotavirus antibody and a fluorescently labeled donkey anti-rabbit IgG secondary antibody (Invitrogen). Virus titers were calculated from dilutions that gave countable numbers of foci (20 to 200) per well. Differences in virus titers between cells lines were compared using a two-tailed Student *t* test.

Nucleotide sequence accession number. The VP8* sequence of neonatal G10P[11] N1509 was deposited in GenBank and assigned the accession number KC807203.

RESULTS

The VP8* sequence of P[11] rotaviruses is distinct in the glycan binding domain. Many animal rotaviruses are known to bind glycans with a terminal Sia residue on cell surface glycans. The crystal structures of the VP8* of animal rotaviruses RRV and CRW-8 with bound Sia have been determined, and the residues

TABLE 1 GST-VP8* P[11] shows the highest binding to glycans containing Galβ1-4GlcNAc[a]

Glycan	Chart no.	No. of LacNAc residues	Average rank[b]
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp19	582	>6	83.18
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp25	569	>6	78.09
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp25	566	>6	77.88
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-Sp0	163	3	9.03
Fucα1-2**Galβ1-4GlcNAc**β1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-Sp0 (H type II)	75	3 (terminal fucose)	25.61
Neu5Acα2- 3**Galβ1-4GlcNAc**β1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-Sp0	259	3 (terminal Sia)	5.60
Neu5Acα2-6**Galβ1-4GlcNAc**β1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-Sp0	333	3 (terminal Sia)	3.27
Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ-Sp0 (GD1a-like)	413	0	0.95
Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ-Sp0 (GM1)	145	0	0.59

[a] The strength of binding to glycans with Galβ1-4GlcNAc (in boldface and underlined) was much higher than that of GM1- and GD1a-like glycans that are known partners for some rotavirus strains. The raw data from the glycan array experiments are provided in Table S1 and Fig. S1 in the supplemental material.
[b] Average rank of representative glycans for binding with GST-VP8* P[11] from human neonatal strain N155 calculated from 4 different arrays. The rank for each glycan was calculated using the formula 100 × (test glycan RFU/highest number of RFU in that assay).

that interact with Sia have been identified (2, 4). Studies on a human rotavirus (HAL1166) have shown that VP8* binds A-type HBGA in the same location as Sia in the VP8* of the animal viruses (7). An insertion in amino acid position 187 of HAL1166 VP8* results in a change in the side chain orientation of the conserved residues R101 and Y188, which sterically hinders Sia binding in this pocket but allows A-type HBGA binding (7). The VP8* domains of neonatal P[11] strains (N155 and N1509) show significant alterations in the residues that interact with either Sia in the VP8* domain of animal rotaviruses or the A-type HBGA in the VP8* domain of HAL1166 (Fig. 1). In P[11] rotaviruses, R101 is replaced by a phenylalanine, while the conserved Y188 and Y189 residues are not seen. The VP8* crystal structures of two other human rotavirus strains representing the globally prevalent P[8] (Wa) and P[4] (DS-1) genotypes have also been reported previously (2, 5, 6). Using nuclear magnetic resonance, the Wa VP8* was shown to bind gangliosides, such as GM1, with internal Sia residues. Although the structural determinants of binding to the internal Sia residues are not characterized for these human viruses, the VP8* sequence of P[11] strains varied from the VP8* sequences of these strains (Fig. 1). These sequence comparisons suggest that the amino acid sequence of the VP8* glycan binding domain of P[11] rotavirus strains is distinct from that of the other rotavirus strains whose glycan partners have been characterized and that VP8* P[11] could potentially bind previously uncharacterized glycan partners.

VP8* P[11] binds glycans with Galβ1-4GlcNAc. Potential glycan partners for VP8* P[11] were screened using a glycan array comprising >600 glycans. GST-VP8* P[11] showed distinct binding to a number of glycans, including structures typically found as both N- and O-linked glycans. Overall, the highest rank was seen for glycans containing the Galβ1-4GlcNAc motif (*N*-acetyllactosamine; also called LacNAc), in particular, for glycans with multiple units of LacNAc (polylactosamine). Binding to polylactosamine glycans was seen for GST-VP8* from all P[11] viruses tested, including N155, N1509, and B223 (Table 1; see Table S1 and Fig. S1 in the supplemental material). Previously known rotavirus binding partners, such as GM1 and GD1a-like glycans, showed much lower binding (Table 1). The GST-VP8* P[11] bound with high affinity to the polylactosamine of N-glycans with no preference for the number of branches (Table 1). The strength of binding was, however, directly related to the length of the polylactosamine and less dependent on the terminal monosaccharide. As seen in Table 1, GST-VP8* P[11] bound with less affinity to the shorter, linear glycans on the array. The addition of other monosaccharides to polylactosamine had marginal effects on binding. As can be seen from the glycans listed in Table 1, the presence of an α-1,2-linked fucose (H type II glycan) resulted in an increase in the strength of binding to glycans with shorter polylactosamine chains, whereas the presence of Sia (Neu5Ac, α-2,3, or α-2,6 linkage) resulted in a reduction in binding strength. In addition to N-glycans, strong binding to polylactosamine was also seen, which is typical of O-glycans. Thus, the glycan array results suggest that VP8*P[11] can bind to extended polylactosamine glycans, which are the precursor for H type II HBGAs (1). H type II HBGAs are formed by the addition of a terminal fucose to LacNAc through the activity of α-1,2-fucosyltransferase. The glycan array results also indicate that VP8*P[11] can bind H type II derivatives, while binding is clearly reduced by the addition of Sia to LacNAc. Binding to polylactosamine glycans was not seen for GST-VP8* from SA11-4F or Wa (see Table S1 and Fig. S1 in the supplemental material). GST by itself does not bind to the glycans on the array (data not shown).

GST-VP8* P[11] hemagglutinates adult and cord blood erythrocytes. H type II glycans are present on the surface of O-type RBCs and serve as the precursor for the synthesis of A- and B-type blood group antigens through the activity of glycosyltransferases (1). Thus, hemagglutination assays serve as a useful tool to assess the specificity and biological significance of the glycan array data. In hemagglutination assays with RBCs from adults, GST-VP8* from the P[11] strains as well as SA11-4F hemagglutinated type O, type A, and type B RBCs. The Norwalk virus VLP control

TABLE 2 GST-VP8* P[11] hemagglutinates adult and cord blood erythrocytes RBCs

Blood type or source	Reciprocal HA titer[a]							
	SA11-4F	Wa	HAL1166	N155	N1509	B223	GST	NV VLPs
O	>128	0	0	>128	>128	>128	0	>128
A1	>128	0	>128	>128	>128	>128	0	>128
A2	>128	0	>128	>128	>128	64	0	>128
B	64	0	0	>128	128	64	0	0
Umbilical cord	>128	0	0	>128	>128	>128	0	0

[a] Reciprocal hemagglutination (HA) titers of adult human type O, type A, and type B RBCs as well as pooled cord blood erythrocytes by GST-VP8* proteins. No hemagglutination is indicated by 0. Recombinant Norwalk virus (NV) VLPs, HAL1166 GST-VP8* P[14], and GST were included as controls. GST-VP8* P[11] hemagglutinated all RBCs tested.

hemagglutinated type O and A RBCs but not type B RBCs, while GST-VP8* P[14] from HAL1166 hemagglutinated only type A RBCs, as previously shown (7, 31). No hemagglutination was observed with GST-VP8* Wa or the GST control (Table 2). However, glycan expression on RBCs is developmentally regulated (1). Unbranched polylactosamine chains are more highly expressed on the surface of embryonic and cord blood erythrocytes, while the adult levels of branched chains are reached by about 18 months of age. Therefore, hemagglutination assays were also carried out with pooled umbilical cord blood erythrocytes. GST-VP8* P[11] hemagglutinated umbilical cord blood erythrocytes. Partial hemagglutination of cord blood erythrocytes was seen at the highest concentration of Norwalk virus VLPs, while GST-VP8* P[14], GST-VP8* Wa, and the GST controls showed no hemagglutination (Table 2). These data are consistent with the findings of the glycan array that VP8* P[11] can interact with H type II glycans on cell surfaces.

The VP8* from the SA11-4F and P[11] strains binds to different glycans on RBCs for hemagglutination. SA11-4F is known to bind terminal Sia on cell surfaces, and neuraminidase treatment of RBCs resulted in a complete loss of hemagglutination. In contrast, neuraminidase treatment resulted in an increased hemagglutination titer for VP8* P[11] from N155 and B223 (Table 3), indicating that in untreated RBCs, Sia may be masking additional sites for GST-VP8* P[11] binding.

GST-VP8* P[11] binds all CHO cells, but infectivity is enhanced with expression of H type II. The specificity and biological relevance of glycan array data were further assessed by binding and infectivity assays on CHO cells that differ in expression of HBGA. Binding experiments on parental, H-type, and A-type CHO cells showed similar binding patterns using GST-VP8* P[11] (Fig. 2). There was no significant difference in the expression of polylactosamines between these cell lines (Fig. 3). However, a significant increase in human and bovine G10P[11] titer was observed in all CHO cells that express the enzyme Fut2, including the double-transfectant CHO cells that express Fut2 and A- or B-type glycosyltransferases, compared to that observed in the parental cells (Fig. 4). The enzyme Fut2 catalyzes the addition of a terminal fucose in α-1,2 linkage to the LacNAc precursor. The additional modification of the H type II to A-type or B-type HBGA did not result in any further significant increase in virus titer, indicating that the expression of the H type II was the critical factor for increased infectivity. Terminal Sia binding animal rotavirus strain SA11-4F, internal Sia binding Wa, and A-type HBGA binding HAL1166 were included as controls in the infectivity experiments. No difference in infectivity between the cell lines was observed for SA11-4F and Wa, while HAL1166 showed significantly enhanced infectivity on A-type cells. These data confirm that the interaction of VP8* P[11] with type II glycans on cell surfaces is biologically relevant.

TABLE 3 Neuraminidase treatment of RBCs results in increased hemagglutination with GST-VP8* P[11]

Virus strain	Reciprocal HA titer[a]	
	Without neuraminidase	With neuraminidase
SA11-4F	512	0
N155	512	>2,048
B223	256	>2,048
HAL1166	0	0

[a] Reciprocal hemagglutination (HA) titers of adult human type O RBCs by GST-VP8* proteins following neuraminidase treatment. No hemagglutination is indicated by 0.

Neuraminidase treatment of cells results in enhanced binding on CHO cells. CHO cells express a large number of sialylated LacNAc glycans (32). Removal of the terminal Sia should therefore result in increased binding on these cells. Indeed, neuraminidase treatment of CHO cells resulted in significantly enhanced binding for GST-VP8* P[11], while no significant difference was observed with GST-VP8* P[14] (Fig. 5).

Bovine rotavirus-derived VP4 is critical for increased infectivity. The VP4 in the human neonatal P[11] rotaviruses are of bovine rotavirus origin (20, 24). To further confirm that the increased infectivity with H type II CHO cells was directly mediated by the bovine rotavirus-derived P[11] VP4, infectivity assays were carried out on parental and single-transfectant H type II CHO cells using SA11-4F, bovine G10P[11] rotavirus B223, and six reassortants of these two parental viruses. B223 shows nearly 94% identity in the VP4 gene with the VP4 of the bovine-human reassortant G10P[11] strains. Infections with B223 and reassortant viruses R-491, R-004, and R-198 that express the bovine P[11] VP4 resulted in significantly higher virus titers in cell lines expressing H type II than in parental cells (Fig. 6), confirming that the bovine-origin P[11] VP4 is the key mediator of this virus-cell interaction.

DISCUSSION

Rotavirus infections in neonates are clinically and epidemiologically distinct from infections in older children (11). They are caused by unusual virus strains that appear to be geographically restricted and show remarkable strain stability in comparison to the diversity of strains seen in older children. It has been speculated that the age-dependent restriction of unusual viruses for neonates could be mediated by the interaction of the spike protein with maturation-dependent host components (33); however,

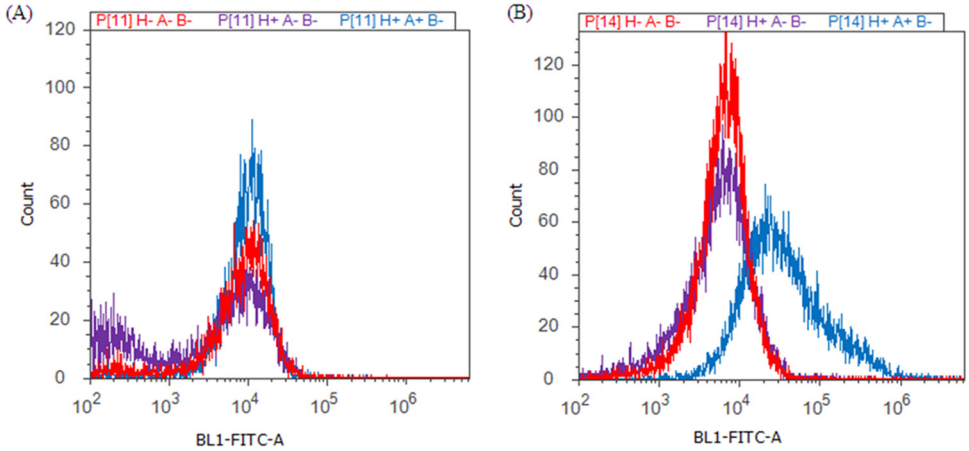


FIG 2 GST-VP8* P[11] binds all CHO cell types. (A and B) Binding of FITC-GST-VP8* P[11] and P[14], respectively, to parental CHO cells (H−/A−/B−, red), the single-transfectant cells expressing the Fut2 enzyme (H+/A−/B−, purple), and the double-transfectant cells with both Fut2 and A-type glycosyltransferase (H+/A+/B−, blue).

there has been little conclusive evidence for this to date. This is the first report demonstrating that the VP8* of a clinically important neonatal rotavirus strain binds to Galβ1-4GlcNAc, the precursor for type II glycans, and that this binding is biologically relevant for infection. The binding of P[11] VP8* to precursor glycans raises important questions about how modification of glycans during the course of neonatal development may alter susceptibility to infectious agents.

The repertoire of studies on microbes and their glycan partners has expanded with recent technological advances in the field of glycobiology. There is increasing evidence to suggest that glycan-pathogen interactions play a key role in the pathogenesis of several infectious agents. This study raises additional questions on whether developmental modification of glycans may also influence pathogenesis. Studies in rodents have demonstrated that glycosylation changes in the gut occur throughout the postnatal phase of development and are controlled by hormonal and dietary factors through the activity of glycosyltransferases (34). However, glycan changes in the human neonatal gut have not been extensively studied. Some studies on the developmental regulation of glycans on human RBCs show that embryonic RBCs have an abundance of unbranched polylactosamine chains and a relative lack of branched chains (1). Linear polylactosamine chains are modified by the activity of the enzyme β1-6 *N*-acetylglucosaminyltransferase, which transfers *N*-acetylglucosamine in β1-6 linkage to internal galactose residues in polylactosamine. This results in the formation of β1-6 *N*-acetylglucosamine branches, which may then serve as the substrate for subsequent LacNAc molecules, thereby forming branched polylactosamine chains. Adult levels of these branched structures are achieved at

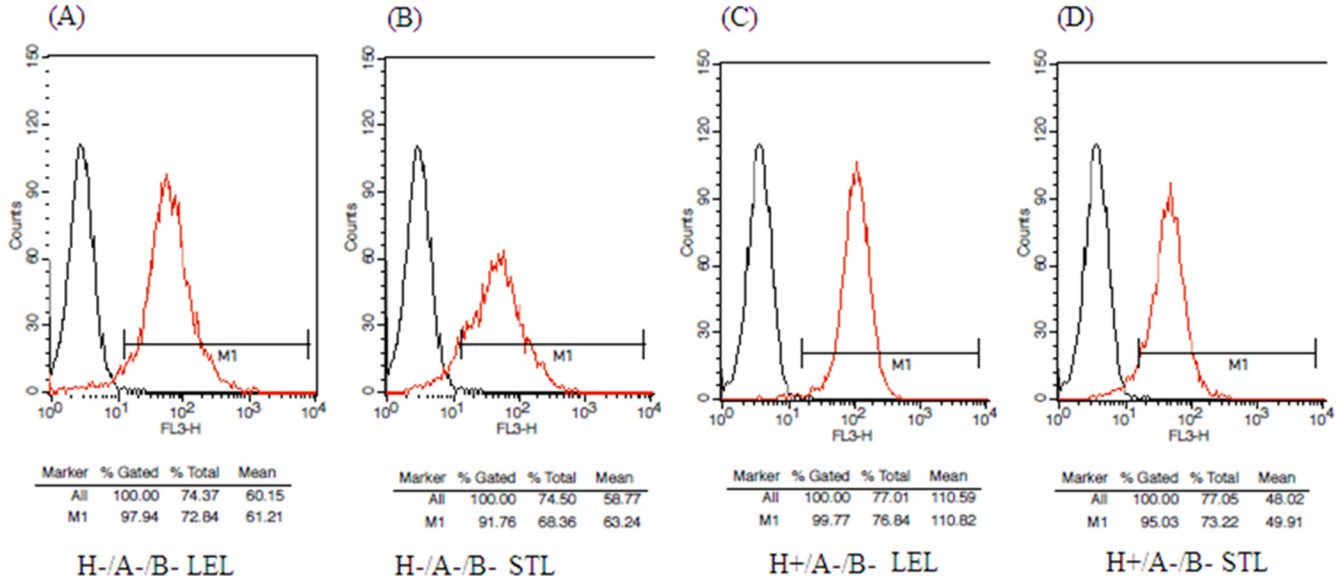


FIG 3 CHO cells do not differ in the levels of polylactosamine. Parental and H-type CHO cells were assayed for polylactosamine by flow cytometry using LEL and STL lectins. (A and B) Parental H−/A−/B− CHO cells assayed using LEL and STL lectins, respectively; (C and D) H-type H+/A−/B− CHO cells assayed using LEL and STL lectins, respectively.

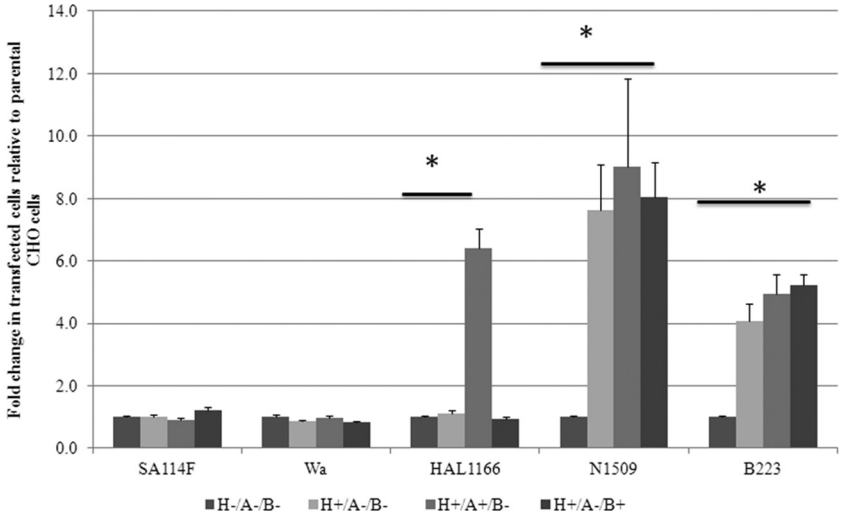


FIG 4 Infectivity of P[11] rotavirus increases with expression of H antigen. The infectivity of rotaviruses in CHO cell lines genetically modified to stably express different glycans is shown. Infection was carried out in parental CHO cells (H−/A−/B−), the single-transfectant cells expressing the Fut2 enzyme (H+/A−/B−), and the double-transfectant cells with both Fut2 and A type glycosyltransferase (H+/A+/B−) or B type glycosyltransferase (H+/A−/B+). The rotavirus strains used (and their titers on CHO cells) include SA11-4F, which binds terminal Sia (4 × 105 FFU/ml, where FFU is focus-forming units); Wa, which binds internal Sia (5.5 × 105 FFU/ml); HAL1166, which binds A-type HBGA (7.7 × 103 FFU/ml); human neonatal P[11] rotavirus strain N1509 (1.5 × 104 FFU/ml); and bovine P[11] rotavirus strain B223 (5.2 × 103 FFU/ml). The *y* axis represents the fold difference in comparison to the results for the parental cells. Error bars represent standard errors of the means of data from quadruplicate wells from at least 3 independent experiments. A *P* value of <0.001 was considered statistically significant and is represented by an asterisk.

about 18 months of age, while unbranched polylactosamine reactivity reaches very low levels. Little is known about such modifications in the human gut during the course of development. It is possible that the polylactosamine is abundantly present in the neonatal gut but is modified during development by the addition of various glycans that results in changes in glycan length or degree of branching. This may in part explain the increased neonatal susceptibility to P[11] rotaviruses and a reduction in the infectivity of these strains beyond the neonatal age group. Interestingly, the globally prevalent human rotavirus strains with P[4] and P[8] VP4 types that infect older children have been identified to bind to the modified HBGAs and not the precursor units (8, 9).

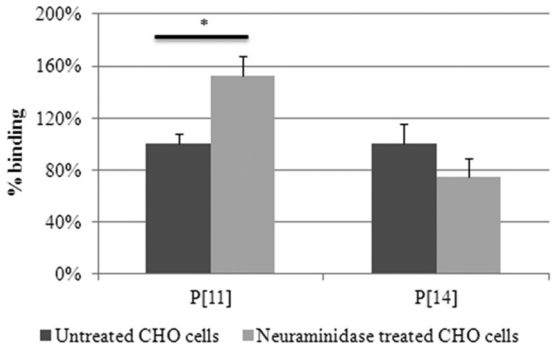
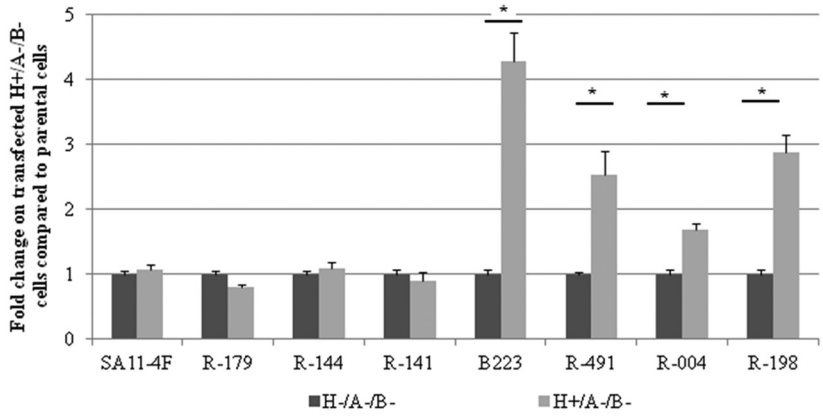


FIG 5 Neuraminidase treatment of CHO cells results in increased binding to GST-VP8* P[11]. Binding of FITC-GST-VP8* P[11] or P[14] on H−/A−/B−, H+/A−/B−, and H+/A+/B− CHO cells was carried out in duplicate with and without neuraminidase treatment. The change in the median fluorescence intensity of binding was compared between untreated and neuraminidase-treated cell lines and expressed as the percent change compared to the results for untreated cells. The data from all cell lines were pooled for each protein and treatment group. Error bars represent the standard error of mean change in binding for all experiments within a group. A *P* value of <0.05 was considered statistically significant and is represented by an asterisk.

The hemagglutination assays, binding studies, and differential infectivity on CHO cells corroborate the findings of the glycan array. GST-VP8* P[11] hemagglutinates both umbilical cord blood and adult RBCs due to its ability to bind both polylactosamine glycans and H type II HBGA. Neuraminidase treatment of RBCs results in increased hemagglutination with GST-VP8* P[11], whereas a complete loss of hemagglutination was seen with GST-VP8* from SA11-4F on these cells, indicating that Sia masks or sterically hinders GST-VP8* P[11] binding to untreated RBCs. In the CHO cell binding experiments, the neonatal G10P[11] virus was able to bind all cell types tested, including parental cells; however, the infectivity was enhanced with the expression of H type II HBGA. The limited infectivity on the parental CHO cells may not be due to the lack of availability of the LacNAc glycans in these cells. It has been demonstrated that the parental CHO cells express an abundance of LacNAc glycans; however, there are few glycans with greater than 3 repeats, and CHO cells grown on a monolayer also express a large number of sialylated LacNAc glycans (32). Removal of sialic acid by neuraminidase treatment results in increased binding on these cells. In addition, lectin binding studies on the parental and transfected CHO cells showed that polylactosamine levels did not change between the parental and transfected cells. This suggests that the increased infectivity observed in the transfected cells is due to the addition of H type II structures and not due to concomitant modifications in other glycan motifs, such as Sia or LacNAc repeats, that could have occurred due to competition between the α-1,2-fucosyltransferase and other glycosyltransferases. As can be seen from Table 1, the best binding of GST-VP8* P[11] occurs in the presence of multiple units of LacNAc, and terminal sialylation of LacNAc chains



Virus Strain	SA11-4F	R-179	R-144	R-141	B223	R-491	R-004	R-198
VP4	SA11-4F	SA11-4F	SA11-4F	SA11-4F	B223	B223	B223	B223
VP7	SA11-4F	B223	B223	SA11-4F	B223	B223	SA11-4F	SA11-4F
Other genes	SA11-4F	SA11-4F	B223	B223	B223	SA11-4F	SA11-4F	B223

FIG 6 Increased infectivity on H-antigen-expressing CHO cells is mediated by VP4. The infectivity of SA11, B223, and laboratory-derived reassortant rotaviruses in parental CHO cells (H−/A−/B−) and cells expressing H antigen (H+/A−/B−) is shown. The origins of the VP4, VP7, and remaining genes in virus strains are listed below the strain. *P* values of <0.001 represent significant differences in virus infectivity between the parental and single-transfectant cell lines and are represented by asterisks.

results in a reduction in the strength of binding to GST-VP8* P[11] compared to that for fucosylated LacNAc (H type II). It should be noted that long polylactosamines terminating with fucose or Sia are not present in the glycan array, and this precludes making a definitive assessment of the strength of their binding to GST-VP8* P[11] in the array compared to that of large polylactosamine glycans lacking these monosaccharides.

In this study, it was seen that bovine rotavirus G10P[11] B223 showed a glycan binding profile and hemagglutination properties similar to those of the human neonatal viruses. G10P[11] rotavirus infections have been widely reported to cause diarrhea in cattle in many parts of the world (13, 15, 35, 36). These strains contain all gene segments of bovine rotavirus origin. The human neonatal G10P[11] infections are, however, caused by bovine-human reassortant strains; these viruses possess a VP4 of bovine rotavirus origin (20, 24). To confirm that the presence of the bovine P[11] VP4 was key for the increased infectivity on H type II CHO cells, infectivity assays were carried out with a bovine G10P[11] rotavirus strain B223 and reassortants derived using B223 and SA11-4F that differ in their genetic makeup. B223 and all reassortant viruses possessing the bovine P[11] VP4 (R-491, R-004, and R-198) showed increased infectivity on H type II cells. The availability of reasssortant rotavirus strains thus allowed confirmation that the P[11] VP4 of bovine rotavirus origin is the key mediator of the interaction with type II glycans described in this study. It should, however, be noted that the magnitude of the fold change was the highest for B223 in comparison to that for the reassortant viruses, indicating a role for other genes and gene combinations in infectivity.

The findings of this study are highly relevant in the context of current rotavirus vaccines in developing countries. Rotavirus diarrhea results in nearly half a million deaths annually; over 80% of these deaths occur in the developing countries of Asia and Africa (37). Currently licensed vaccines that are highly efficacious in developed countries do not appear to be as effective in the developing world (38–40). This has led to the evaluation of new vaccines and alternate immunization schedules, including maternal and neonatal immunizations. One of the new vaccines being evaluated in India is 116E, which is based on an asymptomatic human neonatal G9P[11] rotavirus. This strain also possesses a P[11] VP4 gene of bovine rotavirus origin and has been found to be highly immunogenic (21, 24). The findings of this study suggest that neonatal immunization with this neonatal P[11] virus may prove to be effective, as these strains may be able to replicate more efficiently in the neonatal gut and thus result in better vaccine take. However, this also leads to questions of whether vaccine uptake and response may differ at different ages of vaccination depending on the VP4 genotype of the vaccine virus and glycan profile of the vaccinated infant. These questions will be addressed through future studies involving characterization of glycan interactions for additional rotavirus VP4 types, including the globally prevalent genotypes and those present in vaccine viruses.

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