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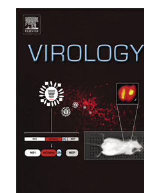

Strain-specific interaction of a GII.10 Norovirus with HBGAs

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ABSTRACT

Noroviruses (NoVs), an important cause of gastroenteritis in humans, recognize human histo-blood group antigens (HBGAs) as receptors. The crystal structures of the protruding (P) domain of a GII.10 NoV (Vietnam 026) in complex with various HBGA oligosaccharides were elucidated. However, the HBGA binding profile of this virus remains unknown. In this study, we determined the saliva and oligosaccharide binding profiles of this virus and the roles of amino acids that are involved in HBGA binding. Our data showed that Vietnam 026 bound to all ABO secretor and non-secretor saliva with clear signals detected by monoclonal antibodies against H3, H1, Le^y, Le^a and sialyl Le^a. Mutagenesis study confirmed the binding site determined by the crystallography study, in which single mutations wiped out the binding function. We also identified amino acids surrounding the central binding pocket that may participate in the binding by affecting the HBGA binding specificity of the GII.10 NoV.

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Introduction

Noroviruses (NoVs), a group of small-and-round structured RNA viruses constituting *Norovirus* genus in the family *Caliciviridae*, are a major cause of epidemic and sporadic acute gastroenteritis in humans. In the United States, NoVs cause19–21 million cases of acute gastroenteritis annually (Hall et al., 2013; Scallan et al., 2011). Structurally NoVs are non-enveloped viruses with protein capsids that encapsulated a single-stranded, positive-sense, polyadenylated RNA genome of ~7.5 kb. The NoV genome contains three open reading frames (ORFs). ORF1 encodes a large polyprotein that is post-translationally cleaved into six nonstructural proteins, while ORF2 and 3 encodes the major (VP1) and the minor (VP2) structural proteins, respectively.

NoV capsid comprises a single major structural protein (VP1) that is divided into two principal domains, the shell (S) and the protruding (P) domains. While the S domain forms the interior, icosahedral shell, the P domain constitutes the protruding spikes extending from the shell (Prasad et al., 1999). The S and the P domains can be structurally and functionally independent. Expression of the S domain alone results in thin layer, icosahedral S particles, corresponding to interior shell of the capsid without the protruding P domains (Bertolotti-Ciarlet et al., 2002; Tan et al., 2004a). On the other hand, the P domain alone can form different complexes, including 24 mer P particles (Tan et al., 2008a; Tan et al., 2011b; Tan and Jiang, 2005b), 12 mer small P particles (Tan et al., 2011a) and P dimers (Tan et al., 2004a; Tan et al., 2008c). These P domain complexes are interchangeable in certain condition (Bereszczak et al., 2012) and they all bind to histo-blood group antigens (HBGAs) (Tan et al., 2011a; Tan et al., 2004a; Tan and Jiang, 2005b), the viral receptors or attachment factors of NoVs (Tan and Jiang, 2005a, 2007, 2010, 2011). NoV P domain complexes have been useful tools for study of NoV–HBGA interactions (Tan et al., 2004a; Tan et al., 2008b; Tan et al., 2006; Tan et al., 2008c; Tan et al., 2009).

NoVs recognize HBGAs in a strain-specific manner and eight HBGA binding profiles of NoVs have been observed (Huang et al., 2003; Huang et al., 2005). HBGAs are complex carbohydrates present abundantly on mucosal epithelia of gastrointestinal track where they serve as receptors or attachment factors for NoVs to initiate infections. In addition, HBGAs also exist as free antigens in biologic fluids, including blood, saliva and milk. Increasing data showed that NoV–HBGA interactions play an important role in the host susceptibility of NoVs (Frenck et al., 2012; Hutson et al., 2002; Lindesmith et al., 2003; Tan and Jiang, 2010, 2011; Tan et al., 2008b). The HBGA binding sites have been mapped on the tops of the P dimers and the structural bases of NoV–HBGA interactions and have been elucidated in detail by crystallography (Bu et al., 2008; Cao et al., 2007; Chen et al., 2011; Choi et al., 2008; Hansman et al., 2011; Kubota et al., 2012; Shanker et al.,

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2011; Shanker et al., 2014) and by site-directed mutagenesis (de Rougemont et al., 2011; Tan et al., 2008c; Tan et al., 2009). These extensive structural studies provide solid foundation to understand the complex interactions between the diverse NoVs and the polymorphic HBGAs, including ABO, Lewis and secretor antigens (Tan and Jiang, 2010, 2011, 2014).

The association of the host susceptibility of NoVs with their HBGA binding profiles has been shown by the human challenge studies on the prototype Norwalk virus (GI.1) (Hutson et al., 2002; Lindesmith et al., 2003) and the two GII.4 NoVs (Frenck et al., 2012). Similar associations have also been shown through outbreak investigations (Carlsson et al., 2009; Kindberg et al., 2007; Tan et al., 2008b; Thorven et al., 2005). In contrast to the predominant GII.4 NoVs, GII.10 NoVs represent a rare genotype. However, a recent crystallography study showed that a GII.10 NoV, named Vietnam 026, interacts with all five secretor HBGAs, including A, B, H, Le^b, and Le^y antigens (Hansman et al., 2011), raising a question on the spectrum of HBGA-binding profile of this NoV and the association of the spectrum with the host susceptibility and prevalence of this NoV. To address these questions we determined the HBGA-binding specificity of Vietnam 026 through conventional saliva and oligosaccharide-based Elisa and confirmed major data of the crystallography studies with minor discrepancies. In addition, we studied the roles of residues in and around the HBGA binding site by structural based mutagenesis analysis and confirmed the binding site determined by the crystallography study. We also observed that residues around the core binding sites may influence the binding specificity of this NoV.

Results

Production of GII.10 P particles

The P domain of Vietnam 026 (GII.10) was expressed as a GST fusion protein (GST–P) in *E. coli*, which yielded ~10 mg/liter bacteria culture. Digestion of the fusion protein by thrombin resulted in a mixture of P protein (~35 kDa) and GST (~26 kDa) (Fig. 1A, lane C). As expected, gel-filtration analysis of the cleaved proteins revealed two major peaks with molecular weight of ~830 kDa and ~50 kDa, respectively (Fig. 1B), each representing the P particles (~830 kDa) and GST dimers (52 kDa) (Fig. 1A). These data indicated that vast majority of the P protein of Vietnam 026 formed P particles.

Hyperimmune sera against Vietnam 026

Such antisera were produced by immunization of the purified P particles to mice ($n=4$, see Materials and methods). Highly reactive, GII.10 NoV antisera with an average titer of ~1:500,000 were obtained

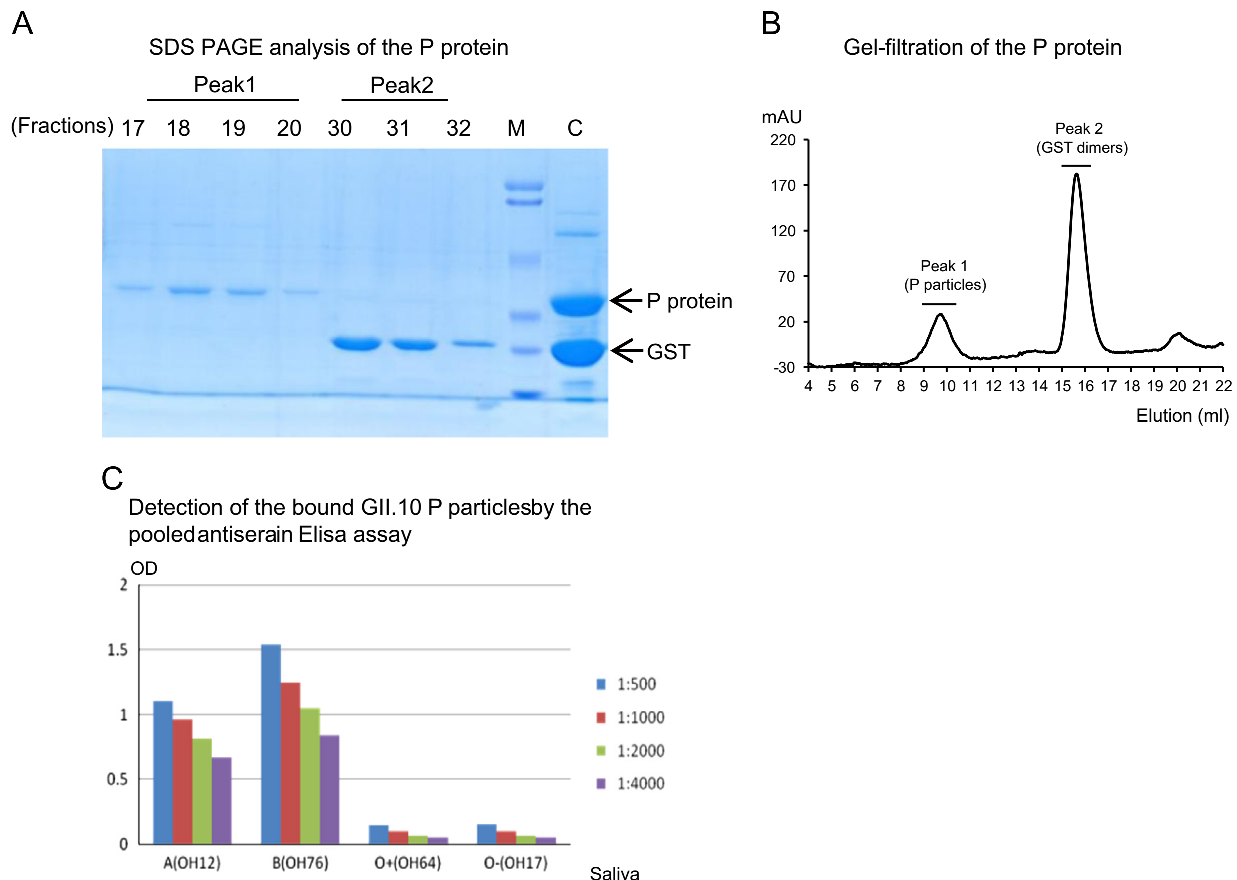


Fig. 1. Production of P particles of Vietnam 026. (A) SDS-PAGE analysis of P proteins. Lane C, the GST–P fusion protein after cleavage by thrombin. Both P protein (~35 kDa) and GST (~26 kDa) are marked with arrows. Fractions of peak 1 and peak 2 were collected from the gel-filtration chromatography (B). Lane M is the prestained protein standards with bands from the top to bottom being 113, 92, 52, 34, 29, and 21 kDa. (B) Gel-filtration analysis of the GST and P proteins using size-exclusion column Superdex 200 (10/300 GL, GE Healthcare Life Sciences). Two major peaks were seen. Peak 1 was near void volume of the column with a molecular weight of ~830 kDa, representing the P particles of the P protein. Peak 2 was corresponding to a molecular weight of ~50 kDa, representing the GST dimers. Four and three fractions of the two peaks were analyzed by SDS-PAGE (A), which revealed P protein and GST, respectively. The gel-filtration columns were calibrated by the Gel Filtration Calibration Kit (GE Healthcare Life Sciences) and the recombinant P particles (830 kDa) of NoV (VA387) and GST dimer of *S. japonicum* (52 kDa). (C) Saliva-based HBGA-binding assays to determine utility and dilution condition of the pooled mouse antisera against the P particles of Vietnam 026. Four saliva samples representing type A (OH12), B (OH76), O secretor (OH64) and nonsecretor (OH17) and four different dilutions (1:500, 1:1000, 1:2000 and 1:4000) of pooled sera were tested. Data were the average value of triplicate experiments. All four saliva samples were Lewis antigen (Le^a/Le^b/Le^x/Le^y) positive.

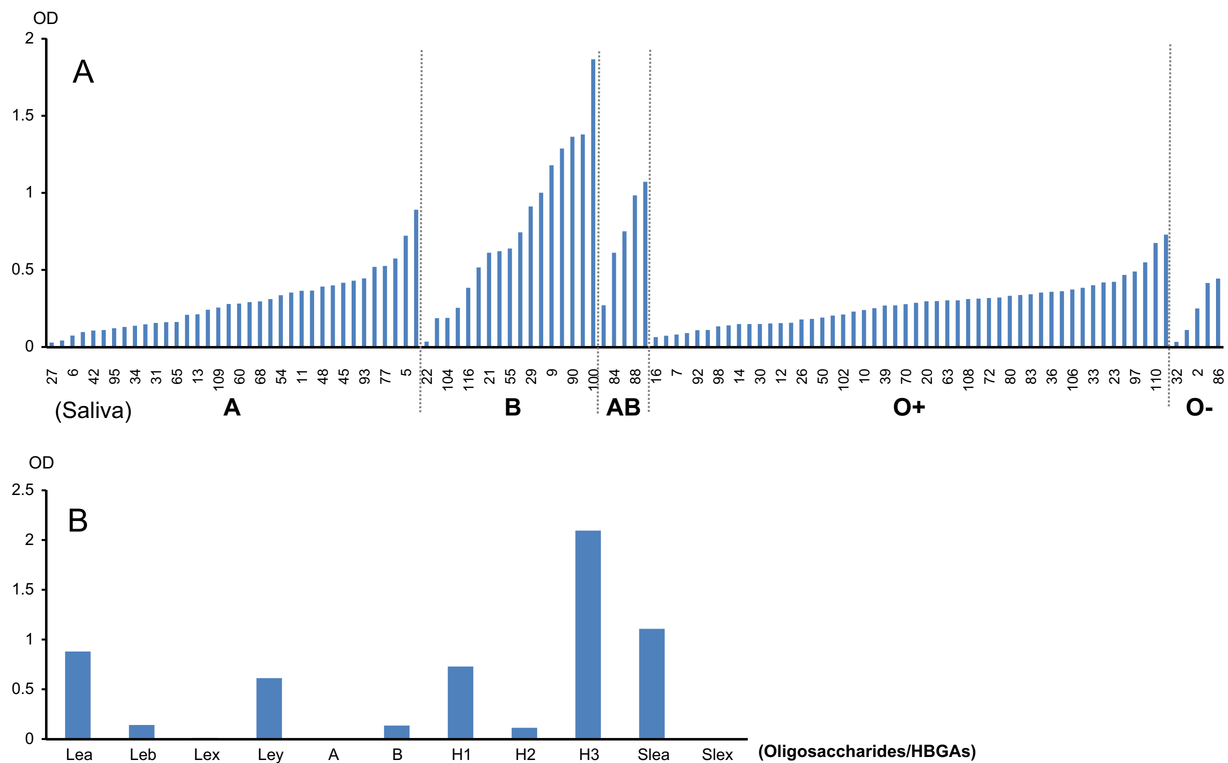


Fig. 2. The binding profile of Vietnam 026 to histo-blood group antigens (HBGAs). (A) Binding of Vietnam 026 P particles to a panel of 113 saliva samples (1:1000) from individuals with different HBGA types. The HBGAs types of the individuals are shown at the bottoms of the panels including type A, B, secretor (O+) and non-secretor (O−). (B) Binding of P particle of Vietnam 026 to a panel of synthetic oligosaccharides representing 11 different HBGAs (H1, H2, H3, A, B, Le^a, Le^b, Le^x, Le^y, SLe^x and SLe^a) (*X*-axis). All oligosaccharides were linked to polyacrylamide (PAA)-biotin as backbones. All data were the average value of triplicate experiments.

(data not shown). The utility and dilution of the pooled antisera for an Elisa assay were determined through saliva-based HBGA-binding assays using four saliva samples representing types A (OH12), B (OH76), O (OH64) secretors and nonsecretor (OH17) with high and low binding capability to the Vietnam 026 P particle (Fig. 1C). The results showed that the pooled antisera at a dilution of 1:2000 were able to detect the bound Vietnam 026 P particles and these conditions were used in all Elisa of this study.

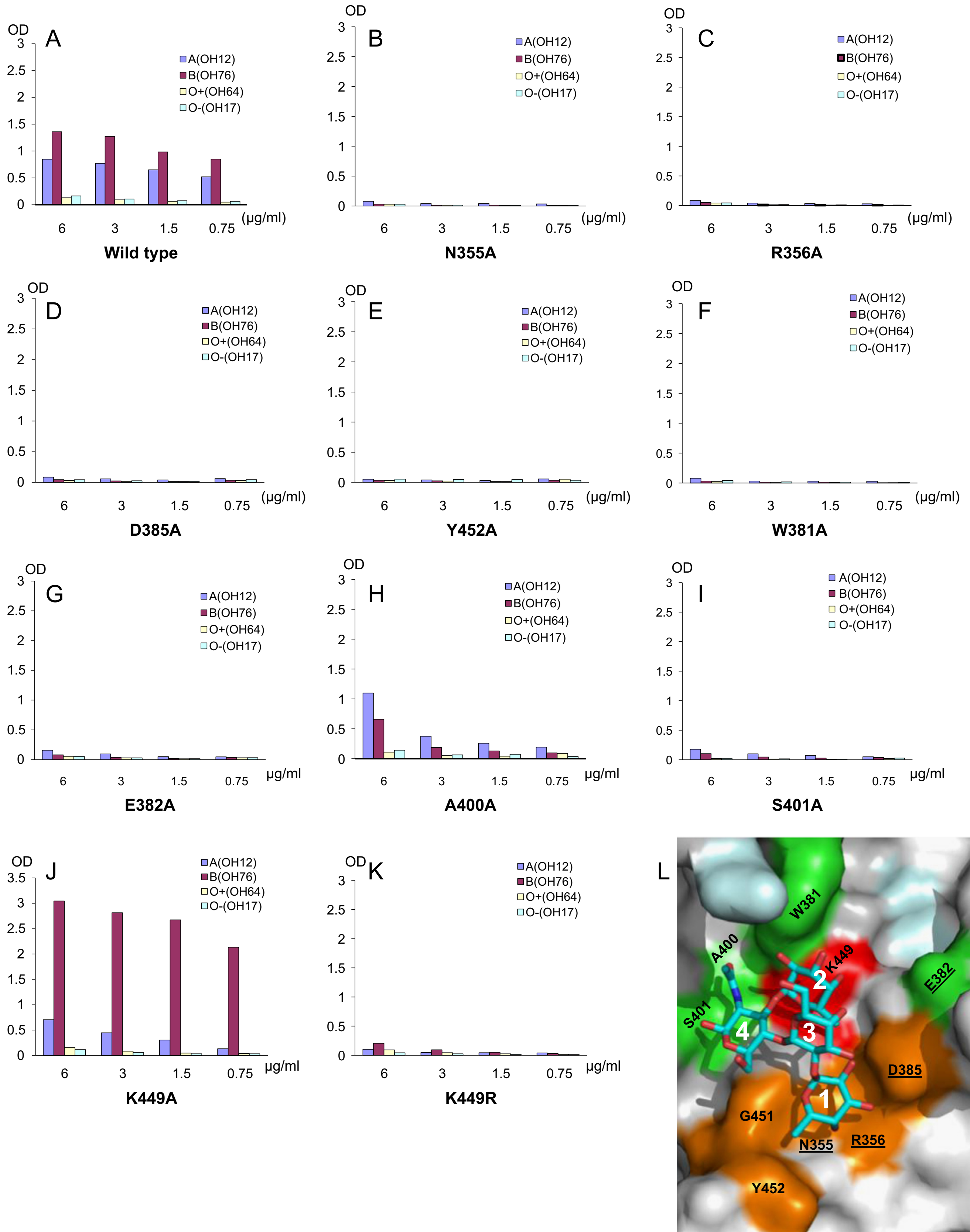
HBGA binding profile of Vietnam 026

We first measured the binding of Vietnam 026 P particles to a panel 113 saliva samples with known blood types that were collected from the general population of Shenzhen, China (Jin et al., 2013).The P particles appeared to bind to salivas of all A, B, O secretors and non-secretors (Fig. 2A), but their binding signals varied, ranging from high to low. This may be due to different levels of A, B, and H antigens in the various saliva samples. Alternatively, HBGAs in the saliva samples other than A, B, H antigens may also play a role (see below). To address this issue, we measured the binding of the P particles to a panel of synthetic oligosaccharides representing 11 different HBGAs. The results showed that the Vietnam 026 P particles bound strongly to H3, sialyl Le^a, Le^a, H1, and Le^y, weakly to B, Le^b, and H2, but not to A, Le^x, and sialyl Le^x antigens (Fig. 2B). These data, together with those from the crystallographic study (Hansman et al., 2011) indicated that the determinants of the binding signals of Vietnam 026 to the saliva samples should include the H (types 1 and 3), Le^a/sialyl Le^a, Le^y, Leb, B, and A antigens. Some discrepancies in binding results of Vietnam 026 using different approaches were noted (see Discussion).

The α-1,2-fucose binding site is critical in GII.10 NoV–HBGA interaction

The crystal structures of the P dimer-HBGA complexes of Vietnam 026 revealed the α-1,2-fucose interacting site as the core binding site of the HBGA binding interface (Hansman et al., 2011) (Fig. 3L). Five amino acids (N355, R356, D385, G451 and Y452) consistently interact directly with the α-1,2-fucose of all six tested secretor oligosaccharides (Fig. 3L, orange region). To further evaluate this site and the roles of the individual residues, alanine mutations were introduced to four of the five residues individually. As expected, all the resulting mutants lost the HBGA-binding function (Fig. 3A–E), indicating the importance of this α-1,2-fucose binding site in interaction with HBGAs and that each individual

Fig. 3. Binding of the wild type P particle of Vietnam 026 and its mutants with single amino acid substitution within the binding interface to saliva samples with different histo-blood group antigens (HBGAs). (A) Binding of the wild type P particle of Vietnam 026 to saliva samples. (B–E) Binding of the mutant P particles with single mutations in the α-1,2-fucose binding site to saliva samples. (F–I) Binding of the mutant P particles with single mutations in the other saccharide-binding sites to saliva samples. (J, K) Binding of the mutant P particles with single mutations at K449 to saliva samples. *X*-axis shows the protein concentration of the P particles and the *Y*-axis indicates the optional densities (OD) at 450 nm. Data were the average values of triplicate experiments. (L) The crystal structure (surface representation) of the HBGA-binding interface of Vietnam 026 (PDB#: 3PA2) with indications of the amino acids that were evaluated by structural-based mutagenesis. The Le^y tetrasaccharide is indicated in stick model, in which the atoms C, O and N are shown in cyan, red and blue, respectively. The four saccharides are labeled as: 1 (α-1,2-fucose), 2 (α-1,3-fucose), 3 (galactose), and 4 (N-acetyl glucoseamine), respectively. The α-1,2-fucose binding site is shown in orange. Amino acids that are involved in interactions with other saccharides are shown in green and red. Underlined amino acids are from the other P domain protomer.


A
OD
A(OH12)
B(OH76)
O+(OH64)
O-(OH17)
(μg/ml)
Wild type
B
N355A
C
R356A
D
D385A
E
Y452A
F
W381A
G
E382A
H
A400A
I
S401A
J
K449A
K
K449R
L
W381
A400
K449
E382
S401
D385
G451
N355
R356
Y452
1
2
3
4

amino acid is indispensable for the structural integrity of this binding site.

Importance of other saccharide-binding sites

Some other amino acids are also involved in interaction with saccharides of the HBGAs other than the α-1,2-fucose, including W381, E382, A400, and S401(Hansman et al., 2011) (Fig. 3L, green region). Mutation of W381, E382 and S401 into an alanine, respectively, abolished the binding function of the three mutants (Fig. 3F, G and I), indicating that these three amino acids are required for the structural integrity of the binding interface. One exception is the A400S mutant that decreased, but remained well-bound to HBGAs (Fig. 3H). This residue is involved in interaction with the Le^y tetrasaccharide through the GlcNAc of the precursor disaccharide (Hansman et al., 2011).

Mutation of K449 changed binding specificity

The K449 interacts with A, B and H, but not to Lewis antigens (Hansman et al., 2011) (Fig. 3L, red region). Interestingly, K449A mutant exhibited a HBGA-binding pattern distinct from the wild type one (Fig. 3, compare A and J), in which K449A mutant decreased binding to saliva sample of OH 12 (type A) significantly but increased binding to OH 76 saliva (type B). However, the K449R mutant lost binding to all saliva samples (Fig. 3K), although both K and R are positive charged amino acids. This data indicate complex roles of the K449 in the binding function and as factor of binding specificity of GII.10 NoV. Oligosaccharide-based HBGA binding assays were performed to further characterize the binding specificity of the K449A mutant. The results (Fig. 4A) showed that the K449A mutant indeed increased binding to B antigen significantly compared with the wild type Vietnam 026 P particles, while it also exhibited decreased binding to H1, Le^a and sialyl Le^a antigen at certain levels (Fig. 4A). To confirm these observations extended saliva-based binding assays were also carried out, which revealed the increased signal intensities of the K449A mutant accumulating to the B and AB type salivas (Fig. 4B). When the results were organized by the signal intensity of the B antigens, consistently high signals of K449A mutant were seen when any B antigen is detected in the saliva sample (Fig. 4C).

Residues around the binding interface contribute to the binding function differently

To further investigate effects of the residues around the binding interface we selected residues A354, A357, H358, G450 within the region of the binding interface, as well as E359 and R299 outside the direct region of the binding interface for mutagenesis (Fig. 5H). Mutations of these residues to an alanine (or serine) individually revealed different effects (Fig. 5). Mutants A354S, H358A and G450A, representing three of the four residues within the region of the binding interface lost their binding to HBGAs (Fig. 5B, D and E), while mutant A357S (Fig. 5C), the remaining residue within the region of the binding interface, did not reduce significantly compared with the wild type P particles. As expected, mutations at the two residues (R229 and E359) that are away from the binding interface (Fig. 5H) into alanine, respectively, did not affect the binding capability of the mutant P particles (Fig. 5F and G). In fact, E359A mutant even showed some increased binding signal compared with the wild type P particles. These data suggested that residues around the binding interface contribute to the binding function differently.

Discussion

Increasing data suggest that NoV–HBGA interactions correlate with the host susceptibility of NoV infection and illness, making it important to determine HBGA-binding profiles of individual strains for epidemiology assessment of NoV diseases. In addition, a recent study showed that H antigen or HBGA-expressing bacteria promoted replication of a human NoV in the BJAB B cell line (Jones et al., 2014), further highlighting the importance of HBGAs in NoV infection and replication. We characterized in this study the strain-specific NoV–HBGA interaction of a GII.10 NoV, Vietnam 026, in which the HBGA-binding profile was determined and the roles of the amino acids in and around the HBGA-binding interface were evaluated. Our data, together with those known previously (Hansman et al., 2011), showed that Vietnam 026, an isolate of the genotype GII.10, bound H3/H1, A, B, Le^y, Le^a and sialyl Le^a antigens. Accordingly, saliva-based binding assays using a panel of saliva samples from 113 individuals also showed interactions of this NoV to salivas of A, B, O secretor and nonsecretor types, although the binding signals varied. These data raised a question on how to interpret the HBGA binding profile of Vietnam 026 to the low prevalence of GII.10 NoVs. The binding signals of Vietnam 026 P particles to saliva samples appeared to be low in general, which might be a factor for the low prevalence of the GII.10 NoV.

The HBGA-binding profile of Vietnam 026 that binds strongly to H3, Le^a/sialyl Le^a, H1, Le^y, and weakly to B, Le^b, and H2 antigens determined by the conventional binding assays in this study is similar but also different compared with the one determined by co-crystallization study (Hansman et al., 2011). The failure to measure the binding of Vietnam 026 P particle to type A oligosaccharide by a binding assay may be due to a problem of the reagent of the type A oligosaccharide. The difference may be due to distinct methodology of the two studies. For example, co-crystallizations of the P dimers with oligosaccharide HBGAs were performed using extremely high concentrations of both the P protein (2–10 mg/ml) and oligosaccharides (30–60 molar folds excess that of the P protein) (Hansman et al., 2011). This experimental setup would record very weak interactions between these two reagents. In fact, using the direct electrospray ionization mass spectrometry (ESI-MS) a recent study showed that the P dimers of VA387, a GII.4 NoV, bound to all 41 tested HBGAs representing all types 1 to 6A, B, H epitopes and Lewis antigens (Han et al., 2012), although conventional saliva- and oligosaccharide-based binding assays recorded only binding to certain secretor antigens (Huang et al., 2003; Huang et al., 2005). Since native NoV infection are normally caused by NoV contaminated food, water or surface, which is known for low dose, the conventional binding assays with low concentration of NoV capsid proteins might be better reflex NoV transmission in the real world which is comparable with the literatures.

The crystal structures of the HBGA-binding interfaces have been solved in three GI (Bu et al., 2008; Choi et al., 2008; Kubota et al., 2012; Shanker et al., 2014) and five GII (Bu et al., 2008; Cao et al., 2007; Chen et al., 2011; Hansman et al., 2011; Shanker et al., 2011) NoVs. The roles of amino acids in and around the HBGA-binding interfaces have also been studied in some of the above NoVs through structure-guided mutagenesis (Bu et al., 2008; de Rougemont et al., 2011; Kubota et al., 2012; Tan et al., 2008c; Tan et al., 2009). In this study, we systematically evaluated 16 amino acids in and around the HBGA-binding interface of a GII.10 NoV (Vietnam 026) for their roles in NoV–HBGA binding. In summary of the data, two general phenomena are seen, (1) conservation of the HBGA-binding interface and (2) flexibility of the HBGA-binding function. The conservation of the HBGA-binding interface is shown by the conserved amino acids constituting the core structure of the central binding pocket (Tan and Jiang, 2014). Any mutation of these amino acids completely abolished the binding function. In

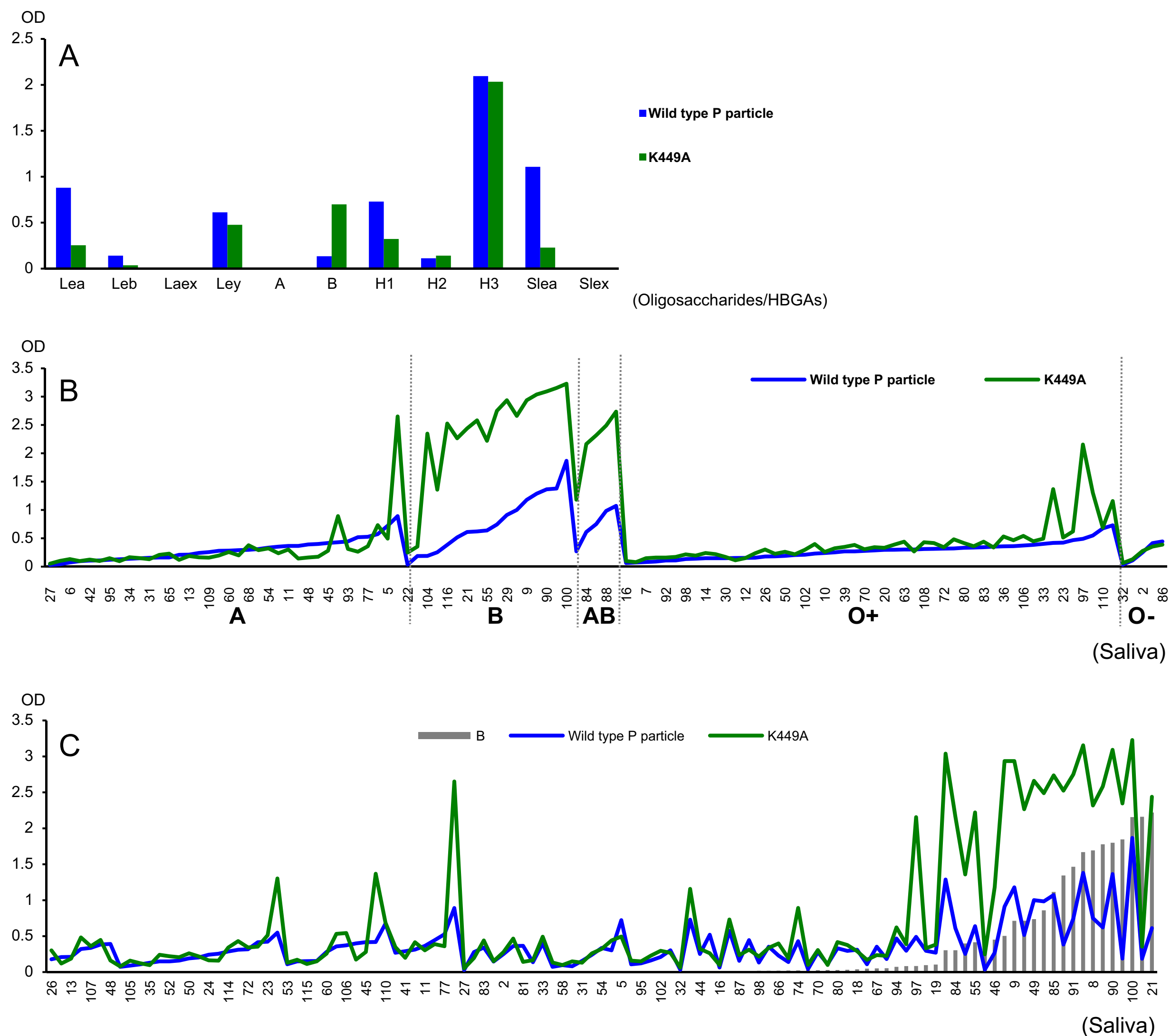


Fig. 4. Binding of Vietnam 026 wild type and K449A mutant to histo-blood group antigens (HBGAs). (A) Binding of the wild type and K449A mutant P particles to a panel of synthetic oligosaccharides representing 11 different HBGAs (H1, H2, H3, A, B, Le^a, Le^b, Le^x, Le^y, SLe^x and SLe^a) (*X*-axis). All oligosaccharides were linked to polyacrylamide (PAA) with biotin. (B) Binding of the wild type and K449A mutant P particles to a panel of 113 saliva samples (1:1000) from individuals with different HBGA types. The HBGAs types of the individuals are shown at the bottoms of the panels including type A, B, secretor (O+) and non-secretor (O−). (C) Data from (B) were organized according to the ascending of the signal intensity of B antigens. All data were average values of triplicate experiments.

strain Vietnam 026, amino acids R356, D385, W381, and E382 are these conserved residues which are essential for HBGA binding function and therefore for viability of the strain. On the other hand, the flexibility of the HBGA-binding function is shown by the fact that mutations of certain amino acids around the core structure of the binding sites change the HBGA binding profiles of the mutants. The residue K449 of Vietnam 026 is such a flexible amino acid. The flexibility could potentially allow NoV mutants to establish replications in new target populations. It should also be noted that the mutant K449R lost its binding completely, suggesting that different mutations at the same amino acid could lead to different outcomes, making the conservation/flexibility features more complicated.

In addition to the amino acids that interact directly with the HBGAs as revealed by the crystallography (Hansman et al., 2011), we have identified three other amino acids (A354, A357 and H358) that are not directly involved in the interaction with HBGAs, but play an important role for HBGA-binding function. Mutation of these residues to an alanine or serine wiped out the binding function completely. Thus, these residues may contribute to the conformational integrity of the HBGA binding interface.

In summary, our study established the HBGA-binding profile of a rare GII.10 NoV, providing new information to the relationship between the HBGA-binding property and the epidemiology outcome of NoVs. In addition, the systematic analysis of the individual residues in and around HBGA binding site shed light into the complex interaction between the diverse NoVs and the polymorphic HBGAs.

Materials and methods

Expression constructs of wild type and mutant P particles of Vietnam 026

The P domain-encoding cDNA sequences of Vietnam 026 (GII.10) (GenBank Accession no.AF504671, amino acid sequences from 222 to 557 of VP1 were used) were synthesized (GenScript, Piscataway, NJ) and cloned into the expression vector pGEX-4T-1 (GE Healthcare Life Sciences) between the BamHI and Not I sites. A cysteine containing short peptide (CDCRGDCFC) was added to the C-terminal end of the P domain to facilitate the P particle formation as described previously (Tan et al., 2008a; Tan and Jiang, 2005b). Mutant P particles with a single amino acid mutation at the HBGA binding interface (Table 2) or

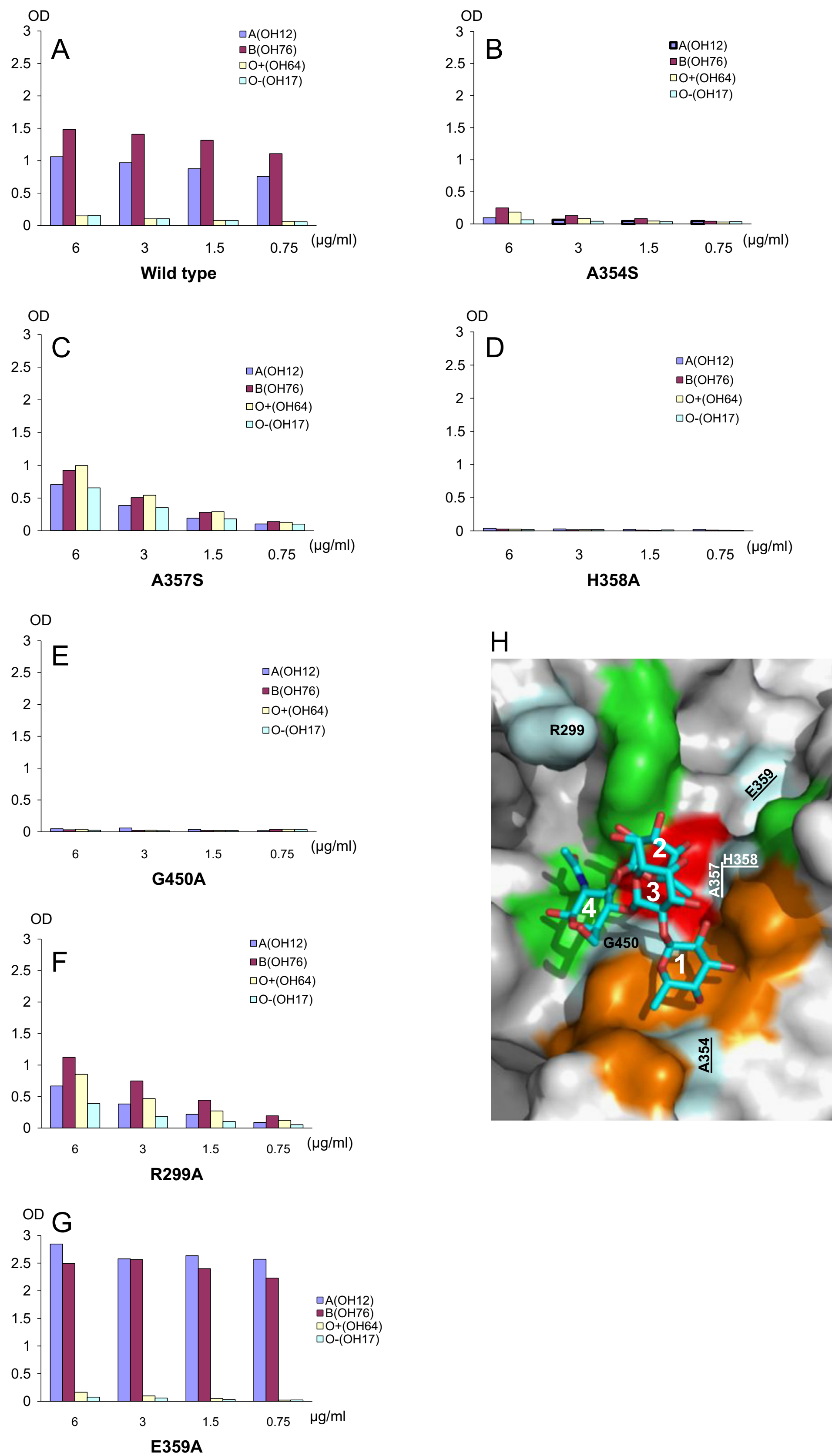


Fig. 5. Binding of the wild type P particle of Vietnam 026 and its mutants with single amino acid substitution within or around the binding interface to saliva samples with different histo-blood group antigens (HBGAs). (A) Binding of the wild type P particle of Vietnam 026 to saliva samples. (B–E) Binding of the mutant P particles with single mutations within the HBGA-binding interface to saliva samples. (F, G) Binding of the mutant P particles with single mutations around the HBGA-binding interface to saliva samples. (H) The crystal structure (surface representation) of the HBGA-binding interface of Vietnam 026 (PDB#: 3PA2) with indications of the amino acids within or around the HBGA-binding interface. The Le^y tetrasaccharide is indicated in stick model, in which the atoms C, O and N are shown in cyan, red and blue, respectively. The four saccharides are labeled as: 1 (α-1,2-fucose), 2 (α-1,3-fucose), 3 (galactose), and 4 (N-acetyl glucoseamine), correspondingly. The labeled amino acids are not directly involved in interactions with HBGAs. The residues that are involved in HBGA-binding directly are shown in orange (α-1,2-fucose binding site), green and red. Underlined amino acids are from the other P domain protomer.

Table 1
Primers used for site-directed mutagenesis to generate single amino acid mutation.

#	Name	Primer sequence (5′ to 3′)	Sense	Mutation
1	P1742	GGTAACCTGCCGGCTGCCCGTGCTCACGAAGCTG	+	N355A
2	P1743	CAGCTTCGTGAGCACGGGCAGCCGGCAGGTTACC	−	N355A
3	P1744	GGTAACCTGCCGGCTAACGCTGCTCACGAAGCTG	+	R356A
4	P1745	CAGCTTCGTGAGCAGCGTTAGCCGGCAGGTTACC	−	R356A
5	P1746	ATCCAGTTCTCTACCGCGGAAACCCAGGACGTTTC	+	W381A
6	P1747	GAAACGTCCTGGGTTTCCGCGGTAGAGAACTGGAT	−	W381A
7	P1748	CAGTTCTCTACCTGGGCAACCCAGGACGTTTCTTC	+	E382A
8	P1749	GAAGAAACGTCCTGGGTTGCCCAGGTAGAGAACTG	−	E382A
9	P1750	ACCTGGGAAACCCAGGCCGTTTCTTCTGGTCAG	+	D385A
10	P1751	CTGACCAGAAGAAACGGCCTGGGTTTCCCAGGT	−	D385A
11	P1752	ACCCCGGTTGGTCTGTCTTCTGTTGACGCTAAC	+	A400S
12	P1753	GTTAGCGTCAACAGAAGACAGACCAACCGGGGT	−	A400S
13	P1754	CCGGTTGGTCTGGCTGCTGTTGACGCTAACTCTC	+	S401A
14	P1755	GAGAGTTAGCGTCAACAGCAGCCAGACCAACCGG	−	S401A
15	P1756	TCTTTCATCCCGCTGGCAGGTGGTTACGGTAAC	+	K449A
16	P1757	GTTACCGTAACCACCTGCCAGCGGGATGAAAGA	−	K449A
17	P1758	ATCCCGCTGAAAGGTGCTTACGGTAACCCGGC	+	G451A
18	P1759	GCCGGGTTACCGTAAGCACCTTTCAGCGGGAT	−	G451A
19	P1760	CCGCTGAAAGGTGGTGCCGGTAACCCGGCTATC	+	Y452A
20	P1761	GATAGCCGGGTTACCGGCACCACCTTTCAGCGG	-	Y452A

Table 2
Summary of the systematic mutagenesis analysis on residues in and around the HBGA-binding interface of NoV strain Vietnam 026.

Mutants[a]	Relative binding affinity to HBGAs[b] P particles[c]			
	O+	O−	A	B
Wild type	−	−	+	+
N355A*	−	−	−	−
R356A*	−	−	−	−
A357S	++	++	++	++
D385A*	−	−	−	−
K449A*	−	−	+	++++
K449R*	−	−	−	−
G451A*[&]	ND	ND	ND	ND
Y452A*	−	−	−	−
W381A*	−	−	−	−
E382A*	−	−	−	−
S401A*	−	−	−	−
A400S*	−	−	++	+
A354S	−	−	−	−
H358A	−	−	−	−
E359A	−	−	++++	++++
R299A	+	++	++	+++

[a] Amino acids with a star symbol are predicted to interact with Le^y and Le^b tetrasaccharide, H-2, Leb A- and/or B trisaccharides and H-2 disaccharide by crystallographic study.
[b] Number of "+" indicated the relative binding affinity of the mutant P particles to HBGAs. "−" indicated a complete loss of binding.
[c] The P particle formation of wild type and all mutants were confirmed by gel-filtration.
[&] ND: not determined.

surrounding area were constructed based on the wild type construct as template through site-directed mutagenesis approach using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the corresponding primers (Table 1) as described elsewhere (Tan et al., 2008c; Tan et al., 2009). The mutations were confirmed by DNA sequencing.

Production of wild type and mutant P particles of Vietnam 026

The wild type and mutant P particles were expressed and purified as described previously (Tan et al., 2004a; Tan and Jiang, 2005b). Briefly, after sequence confirmation the P proteins were expressed in *E. coli* strain BL21 with IPTG (0.5 mM) induction at room temperature (~25 °C) overnight. The P protein–GST fusion proteins were purified using the Glutathione Sepharose 4 flow (GE Healthcare Life Sciences) according to the manufacturer's protocol. The P proteins were released from GST by thrombin (GE Healthcare Life Sciences) digestion. The formation of the P particle was determined by gel-filtration using a size-exclusion column Superdex 200 (GE Healthcare Life Sciences) powered by an AKTA-FPLC system (model 920, GE Healthcare Life Science), in which the P particles formed a peak at ~830 kDa and the P dimer at ~69 kDa, respectively (Tan et al., 2004a; Tan and Jiang, 2005b; Tan et al., 2006). All mutant P particles that were created in this study with single residue mutation at or near the HBGA binding interface do not change their P particle formation as shown by gel-filtration chromatography (see below).

Production of GII.10 mouse antisera

The purified wild type P domain protein (P particles) of Vietnam 026 was immunized to mice intranasally without adjuvant at a dose of 25 μg/mouse (N=4) four times at two-week intervals. Sera were prepared from bloods that were collected before the first immunization and two weeks after the final immunization. These antisera were used for detection of Vietnam 026 P particle that bound to the coated HBGAs (see below).

Gel-filtration chromatography

This was performed through an Akta Fast Performance Liquid Chromatography (FPLC) system (model 920, GE Healthcare Life Sciences) using a size exclusion column (Superdex 200, 10/300 GL with 24 ml bed volume, GE Healthcare Life Sciences), as described previously (Tan et al., 2004a; Tan and Jiang, 2005c; Tan et al., 2004b). The columns were calibrated using gel-filtration calibration kits (GE Healthcare Life Sciences) and the purified NoV P particle (~830 kDa) of NoV VA387 (Tan and Jiang, 2005c) and GST dimer of *S. japonicum* (~50 kDa). The protein identities in the peaks of interest were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and protein concentration determination

Recombinant proteins were analyzed by SDS-PAGE using freshly prepared 10% separating gels. Protein concentrations were determined on SDS-PAGE using diluted bovine serum albumin (BSA, Bio-Rad) with known concentration as standards.

HBGA binding assay

The saliva- and synthetic-oligosaccharide based binding assays were performed basically as described previously (Huang et al., 2003; Huang et al., 2005). The affinity-column purified P particles were first diluted to 0.5 mg/ml as starting solutions, then they were diluted further in a 2-fold-series to 6 μg/ml, 3 μg/ml, 1.5 μg/ml and 0.75 μg/ml directly on the Elisa plates, on which saliva- or synthetic-oligosaccharides have been coated. The saliva samples used in this study were well-defined in our laboratory (Huang et al., 2003; Huang et al., 2005). The synthetic oligosaccharide-based binding assays were performed using a panel of oligosaccharides representing 10 different HBGAs (B, H1, H2, H3, Le^a, Le^b, Le^x, Le^y, sialyl Le^a and sialyl Le^x) as reported previously (Huang et al., 2003; Huang et al., 2005). The detection antibody was the pooled mouse antisera after immunization with Vietnam 026 P particle (see above) at a dilution of 1:2000. The bound antibodies were detected by HRP-conjugated goat anti-mouse secondary antibody (MP Biomedicals, Inc.)(1:3000). Saliva samples were boiled and diluted 1000×, while the synthetic oligosaccharides were at 2 μg/ml for coating the plates.

Crystal structure visualization and analysis

The crystal structures of the P dimer of Vietnam 026 in complex with different oligosaccharides were analyzed using the PyMOL software (DeLano Scientific LLC, Palo Alto, CA). The PDB files of Vietnam 026 were downloaded from the Protein Data Bank at the Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

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