A Single-Amino-Acid Substitution in the P2 Domain of VP1 of Murine Norovirus Is Sufficient for Escape from Antibody Neutralization

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Noroviruses cause epidemic outbreaks of acute viral gastroenteritis worldwide, and the number of reported outbreaks is increasing. Human norovirus strains do not grow in cell culture. However, murine norovirus (MNV) replicates in the RAW 264.7 macrophage cell line and thus provides a tractable model to investigate norovirus interactions with host cells. Epitopes recognized by monoclonal antibodies (MAbs) against the human norovirus strains Norwalk virus and Snow Mountain virus (SMV) identified regions in the P domain of major capsid protein VP1 important for interactions with putative cellular receptors. To determine if there was a relationship between domains of MNV VP1 and VP1 of human norovirus strains involved in cell binding, epitope mapping by phage display was performed with an MNV-1-neutralizing MAb, A6.2.1. A consensus peptide, GWWEDHGQL, was derived from 20 third-round phage clones. A synthetic peptide containing this sequence and constrained through a disulfide linkage reacted strongly with the A6.2.1 MAb, whereas the linear sequence did not. Four residues in the A6.2.1-selected peptide, G327, G333, Q334, and L335, aligned with amino acid residues in the P2 domain of MNV-1 VP1. This sequence is immediately adjacent to the epitope recognized by anti-SMV MAb 61.21. Neutralization escape mutants selected with MAb A6.2.1 contained a leucine-to-phenylalanine substitution at position 386 in the P2 domain. The predicted location of these residues on VP1 suggests that the phage peptide and the mutation in the neutralization-resistant viruses may be in close proximity to each other and to residues reported to be important for carbohydrate binding to VP1 of human norovirus strains.

Noroviruses cause epidemic outbreaks of viral gastroenteritis in all age groups. Attack rates are high in these outbreaks, and rapid person-to-person spread is common. Epidemiological data clearly define noroviruses as being the most importantetiologic agent of viral gastroenteritis outbreaks worldwide, and recent surveillance data indicate that the number of reported outbreaks is increasing (2, 7, 8, 25, 33, 34). These emerging viruses are genetically and antigenically diverse. There are two predominant norovirus genogroups that infect humans, genogroup I (gI) and gII. More than 16 genotypes with variants within genotype that likely translate into variable antigenic types have been described within these genogroups. Noroviruses are stable on environmental surfaces, easily transmitted by stool and vomitus, and are shed for several days after symptoms resolve (9). Prolonged virus shedding thus results in asymptomatic individuals remaining infectious and a point source for further contamination of food sources or secondary transmission to contacts.

Noroviruses have single-stranded positive-sense RNA genomes that encode three open reading frames (ORFs) (reviewed in reference 12). ORF1 encodes the nonstructural proteins that are processed by a viral 3C-like protease. ORF2 encodes the major structural protein VP1, and ORF3 encodes a minor structural protein, VP2. The capsid is composed of 180 copies of VP1, and the structure of the virus particle has been determined to a resolution of 2 Å (31). VP1 folds into a shell (S) domain and a protruding (P) domain that is further divided into P1 and P2 domains. The P1 domain consists of amino acid residues 225 to 278 and 406 to 520 (Norwalk virus [NV] strain numbering). This region is relatively well conserved among norovirus strains across genogroups. The P2 domain consists of amino acids 279 to 405 as an insertion into the P1 domain, is hypervariable in sequence, and is suggested to be the primary domain that mediates cellular receptor binding. Expression of VP1 in insect cells by recombinant baculoviruses results in the assembly of virus-like particles (VLPs) that can be purified in high yields. The availability of VLPs has contributed substantially to an understanding of norovirus structure (31, 32), immunogenicity and antigenicity (10, 11, 13, 24, 28), and cell binding properties (26, 39) and was critical in determining correlates of susceptibility to infection (17, 19, 22).

Noroviruses that infect humans bind histo-blood group carbohydrates, and susceptibility to infection correlates with the expression of α1,2-fucosyltransferase and the secretor phenotype for the prototype gI NV strain (19, 22, 26). Eight binding patterns have been proposed, consisting of norovirus strains that belong to either the ABH or Lewis type (14, 16, 18, 37). Carbohydrate binding patterns have been defined by the binding of VLPs to blood group antigens on gastrointestinal epithelial cells (26), VLP interactions with saliva from individuals with different phenotypes (14, 16), and blood type-specific hemagglutination assays (19, 20). The latter assays also have been performed with the P domain of VP1 alone, and specific binding patterns were generally retained (35). Several lines of evidence suggest that the P2 domain mediates binding to histo-blood group carbohydrates. Expression of the P domain in...
bacteria results in the formation of dimers or higher-order structures termed P particles that bind carbohydrates in saliva assays with predicted strain specificity (38). Targeted mutations in a predicted binding pocket within P2 abolished carbohydrate binding (36). Epitopes recognized by monoclonal antibodies (MAbs) that block binding to CaCo-2 cells and inhibit VLP-mediated hemagglutination were also mapped to the P2 domain of VP1 for NV (g1.1) and gII.2 Snow Mountain virus (SMV) (24). Most recently, the structure of the P domain of gI1 strain VA387 in complex with blood group trisaccharides revealed protein-carbohydrate contact sites also within the P2 domain (5).

Murine norovirus (MNV) is the only norovirus that replicates in cell culture and thus provides an important model to understand norovirus biology in the context of a native virus infection (21, 40). To investigate domains of MNV-1 VP1 required for interactions with cells and to determine how closely these regions coincide with functional domains of VP1 of human noroviruses, we screened a random nonapeptide phage library with neutralizing anti-MNV-1 MAb A6.2.1. Synthetic peptides reproducing the selected epitope reacted in a dose-dependent manner with the MAB when constrained through an N- to C-terminal disulfide linkage. Neutralization escape mutants selected with MAb A6.2.1 contained a single-amino-acid change in the P2 domain that was associated with resistance to neutralization. Projection of the relevant MNV-1 VP1 amino acids defined here on the NV VP1 structure based on sequence alignments predicted a close spatial relationship between the MNV-1 phage peptide selected by the MAb, the amino acid substitution in the escape mutants, previously defined epitopes of human norovirus strains, and residues suggested to be important for carbohydrate binding for human strains. This study predicts the first epitope on MNV-1 recognized by a neutralizing MAB and suggests that there may be a common cellular recognition site on the capsids of human and murine noroviruses.

MATERIALS AND METHODS

Cells, virus, and antibodies. MNV-1 CW1 P3 (hereafter MNV-1) and anti-MNV-1 MAb A6.2.1 (immunoglobulin G2a [IgG2a]) were kindly provided by H. W. Virgin and C. E. Wobus. MAb A6.2.1 was described previously (40). Murine RAW 264.7 macrophages (ATCC TIB-71) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.5 g/liter glucose, 10% fetal bovine serum (Atlanta Biologicals), 10 mM HEPES, 4 mML-glutamine, 1.5 g/liter sodium bicarbonate, and 1% penicillin-streptomycin. MNV-1 was propagated in RAW 264.7 cells as previously described (40). MAb 61.21 to SMV was described previously (24) and was used as an IgG2a isotype-matched irrelevant antibody.

Phage-displayed oligopeptide library screens. Phage library screens were performed as described previously (24). Briefly, 10^12 PFU of library J404 (4) in 75 µl Tris-buffered saline (TBS) (50 mM Tris-CI [pH 7.5], 150 mM NaCl) were combined with 100 µg MAb A6.2.1 in phage buffer consisting of TBS containing 1 mg/ml bovine serum albumin (BSA) and 0.5% (vol/vol) Tween 20. The phage-MAB mixture was then combined with 100 µg Gammabind Plus Sepharose beads (GE Healthcare) and incubated for 1 h at room temperature. The mixture was loaded onto a 10-ml Polypep chromatography column (Bio-Rad) and washed with phage buffer. Antibody-phage complexes were eluted with 0.1 M glycine (pH 2.2). First-round eluate was amplified by infecting K91 bacterial cells in late log phase in soft agar. Phages were propagated with 0.15 volumes of a solution containing 10% polyethylene glycol 8000 (PEG 8000) and 0.5 M NaCl. Second- and third-round selections were performed in the same way, and third-round titers were determined by plaque assay.

Phage characterization. Two assays were used to identify third-round phages that reacted with the MAB. First, plaque lifts were performed on the third-round population. Nitrocellulose membranes were probed with MAB A6.2.1 at a final concentration of 4 µg/ml in phosphate-buffered saline (PBS)-0.5% BSA. Positive plaques were detected with a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-mouse secondary antibody. Positive plaques were picked from the original plate and amplified.

In a second assay, third-round plaques from the plaque lifts were transferred into 2 ml of 2% yeast-tryptone medium supplemented with 75 µg/ml kanamycin and incubated for 12 h at 37°C. A 1.5-ml aliquot of each culture was centrifuged for 5 min in a microcentrifuge. Equal volumes of 16% PEG 8000 and 5 M NaCl were added to the supernatants, and precipitated phages were pelleted and then suspended in 50 µl of TBS. The phage concentrate was adhered to the wells of a 96-well Immulon-1B plate overnight at 4°C. The wells were blocked with PBS-5% BSA for 45 min at room temperature. MAb A6.2.1 was added to each well at a final concentration of 1.5 µg/ml, and plates were incubated for 2 h at room temperature. The wells were washed three times with PBS-0.05% Tween 20, and 50 µl of a 1:4,000 dilution of peroxidase-conjugated goat anti-mouse IgG was then added to each well. Plates were incubated for 45 min at room temperature. After washing three times with PBS-Tween 20, the reactions were developed by the addition of 100 µl/well citrate buffer with o-phenylenediamine dihydrochloride (OPD) and H2O2 (10 ml 50 mM citrate buffer, 10 mg OPD, and 5 µl 35% H2O2). The reaction was stopped with 50 µl of 2.5 M sulfuric acid, and the absorbance at 490 nm was measured on a Molecular Devices Versamax microplate reader. Phages that showed reactivity above an irrelevant phage control were selected for sequencing.

Phage DNA was purified using the QIAprep Spin M13 kit (QIAGEN), and the nonapeptide insertion region was sequenced with primer J534.3 (5'-GGTTTGTT CGCTTTTCAGACC-3') using the ABI 310 genetic analyzer and Big Dye v3.1 chemistry.

Peptide enzyme-linked immunosorbent assays (ELISAs). The synthetic peptide used in these studies contained the phage-derived consensus sequence flanked by five (N terminus) and seven (C terminus) residues of M13 phage coat protein pIII (CFYSHSWWEDHGQLPVPVKL). The peptide was constrained by the disulfide linkage of terminal cysteines. Disulfide bond formation was confirmed by mass spectrometry (Global Peptide Services). A constrained peptide derived from SMV (CFYSSHWLPAVIDGLPKVPVKL) was described previously (24) and was used as an irrelevant control peptide. Peptides were synthesized with both constrained and linearized epitopes. To convert the constrained peptide to a linear form, 100 µg of peptide was added to 80 µl sterile water, followed by the addition of the reducing agent Tris(2-carboxyethyl)-phosphine HCl to a final concentration of 30 mM. Reaction mixtures were incubated for 30 min at room temperature. Twofold dilutions of constrained or linearized peptide starting at 2 µg/ml were added to a Maxisorp 96-well plate (Nunc) along with the indicated positive and negative controls. The plate was incubated overnight at 4°C, and the wells were then blocked with 5% BSA in PBS for 4 h at room temperature. The wells were probed with peroxidase-conjugated goat anti-mouse secondary antibody (Jackson Scientific) for 45 min at room temperature. The plate was washed with PBS and developed with OPD substrate, and the absorbance was then measured at 490 nm.

Generation of neutralization escape mutants. Neutralization escape mutants were generated as previously described (30). A total of 1 x 10^7 PFU of MNV-1 were incubated for 30 min with a 10-fold molar excess of MAb A6.2.1 (~40 µg) in 1 ml of complete DMEM. This mixture was then used to infect RAW 264.7 cells. The medium was removed, and 1 ml of medium containing 1/10 the original amount of antibody was added. The cells and medium were harvested at 36 h postinfection. Following three freeze-thaw cycles, cellular debris was pelleted by centrifugation for 5 min at 5,000 x g. Virus in the supernatants was amplified by inoculating a 10-cm dish of RAW 264.7 cells with 500 µl of supernatant diluted in 5 ml of complete DMEM. The cells and medium were collected and subjected to three freeze-thaw cycles, and the supernatant was clarified by centrifugation. Second- and third-round selections were performed by combining 20 µg MAb A6.2.1 antibody with 2.5 ml of the first-round virus for the second round and 30 µg antibody with 2 ml of the second round virus for the third round of selection. The infections and amplifications were carried out as described above. Neutralization assays were performed at each amplification step to monitor the resistant phenotype.

Plaque reduction neutralization assay. Plaque reduction neutralization assays were performed with wild-type MNV-1 and third-round escape mutants as previously described (40). Virus populations were diluted in DMEM to 700, 70, and 7 PFU/ml, and 20 µg MAb A6.2.1 antibody was added to each dilution in a total volume of 800 µl. Similar dilutions without antibody were prepared as controls.
Virus-MAB mixtures were adsorbed to RAW 264.7 cells for 30 min at 37°C. Plaques were counted 2 to 3 days postinfection.

**Viral RNA extraction and sequencing.** Viral RNA for reverse transcription-PCR was extracted as previously described (23). Plaques were picked with 9-in. borosilicate glass Pasteur pipettes, transferred into 300 µl complete DMEM, and used to inoculate RAW 264.7 cells. Cultures were harvested by scraping and collecting the cells and medium, performing three freeze-thaw cycles, and then pelleting the cell debris for 5 min at 5,000 × g. Five hundred microliters of supernatant was combined with an equal volume of 16% PEG 8000-0.8 M NaCl. Virus was precipitated at 30 min for 4°C and then centrifuged for 15 min at maximum speed in a microcentrifuge. The pellet was suspended in 300 µl proteinase K digestion buffer (0.1 M Tris-C1 [pH 7.5], 12.5 mM EDTA, 0.13 M NaCl, 1% [wt/vol] sodium dodecyl sulfate). Fifteen microliters of proteins K was added to each sample and incubated for 30 min at 37°C. Equal volumes of 10% cetyltrimethyl ammonium bromide and 4 M NaCl were added, and the samples were mixed by vortexing for 10 s and then incubated for another 30 min at 56°C. The samples were extracted with an equal volume of 1:1 phenol-chloroform (pH 4.3). RNA was precipitated in 2.5 volumes ethanol and 0.2 M sodium acetate and then suspended in 25 µl of RNase-free water.

Reverse transcription was performed with 60 ng of viral RNA template and 2 pmol of the negative-sense oligonucleotide mnv6753(–) (5′-GAACATTTGAGATGGCCGCAGCAGTGACGCTGGCG-3′) that anneals to MNV-1 RNA at nucleotide 6753. The reaction mixture was heated for 5 min at 65°C and then cooled on ice. Four microliters of 5× first-strand buffer (Invitrogen), 2.0 µl of 0.1 M dithiothreitol, and 1 µl Superscript II reverse transcriptase (Invitrogen) were added to the template and incubated for 20 min at 50°C and then for 30 min at 42°C. The cDNA was amplified by PCR using 2 µl of the reverse transcription reaction mixture in standard PfU Turbo DNA polymerase (Stratagene) PCR using the negative-sense primer mnv6753(+) and forward primer mnv5007(+) that anneals to MNV-1 RNA at nucleotide 5007 (5′-GGGATCATTTGAGATGGCCGCAGCAGTGACGCTGGCG-3′). VP1 cDNA fragments were sequenced using an ABI 310 genetic analyzer and Big Dye v3.1 chemistry starting with primers mnv6753(+) and mnv5007(+). Additional primers were designed from this sequence to obtain the complete VP1 sequence. The internal sequencing primers designed were mnv5545(+), a positive-sense primer that anneals at nucleotide 5545 (5′-CTGGTGCAGTCCITTTGGGCATG-3′), and negative-sense primer mnv6201(–) that anneals at nucleotide 6201 (5′-GGCAGCAGGCTTGGCGAGCCGC-3′). Sequences were aligned and analyzed using SeqMan II DNA sequence analysis software (DNASTAR Inc.). Each mutation was verified by at least two independent sequence determinations.

**RESULTS**

A peptide selected by MAb A6.2.1 aligns with residues 327 to 335 in VP1. MAB A6.2.1 recognizes VP1 and neutralizes MNV-1 infection in RAW 264.7 cells (40). The sequences of 20 third-round phage clones selected by MAB A6.2.1 revealed a consensus peptide sequence, GWGEDHQLQ (Fig. 1). Notable conserved residues in the selected sequences include a glutamic acid at position 4 that was present in 70% of the clones, an aspartic acid at position 5 in 80% of the clones, a histidine at position 6 in 60% of the clones, and a leucine at position 9, conserved in 90% of the clones. Four residues in the A6.2.1 peptide aligned with amino acids 327 to 335 in the hypervariable P2 domain of VP1: G327 and then, sequentially, G333, Q334, and L335. This sequence lies immediately adjacent to an epitope on SMV VP1 at positions 318 to 327 that is reactive with a MAB that inhibits SMV VLP-mediated hemagglutination. This epitope is part of a consensus sequence, AP(L/I)GXPD, conserved in all norovirus strains sequenced so far, including MNV-1 (24) (see Fig. 4). Sequence alignments suggest that G327 corresponds to the conserved G residue in the consensus peptide sequence.

MAb A6.2.1 recognizes a structurally constrained synthetic peptide. MAB A6.2.1 recognizes VP1 in an ELISA but not by immunoblot with denatured protein, suggesting the interaction between the MAB and VP1 is conformation dependent (40). To confirm the reactivity of the phage epitope with the MAB, a synthetic peptide containing the peptide flanked by M13 pIII residues was synthesized to yield the sequence CFYSHSGW WEDHGQLGPPVKVL. The peptide was constrained by disulfide linkage through the N- and C-terminal cysteine residues. A6.2.1 reacted strongly with this peptide by ELISA in a dose-dependent manner (Fig. 2). Antibody reactivity was dependent upon the conformational presentation of the peptide because the reactivity was abolished when the constrained peptide was linearized by reducing the disulfide bond. The pIII residues in the peptide were not responsible for binding because an irrelevant constrained peptide of the same length with the same flanking residues (CFYSHWLPAPIDKLGPPVK VLC) did not react with the MAB. These data all are consistent with the reactivity of MAB A6.2.1 with MNV-1 being conformation dependent and suggest that residues in VP1 surrounding the selected amino acid residues contribute significantly to the structural presentation of the epitope.

Neutralization escape mutants reveal a single-amino-acid change in the P2 domain of VP1. Neutralization escape mutants were generated to compare the epitope selected by phage.

**FIG. 1.** Nonapeptides selected from the J404 phage library by anti-MNV-1 MAB. Shown are peptide sequences obtained from 20 third-round phage clones and the consensus sequence derived from the clones. Shaded residues indicate the most conserved residues, and the derived consensus sequence is shown below the alignment.
display to changes in VP1 associated with resistance to the MAb. After three rounds of selection, infectivity was no longer inhibited (Fig. 3). The sequence of the cDNA amplified from the third-round virus population revealed two nucleotide changes relative to the sequence of the starting virus. One mutation was a G-to-U change at nucleotide 1145. This mutation translated to a change from L to F at position 386. Sequence was also obtained from 18 plaques from the third-round population that had been amplified in the presence of MAb. The L386F mutation was present in all of the clones sequenced, whether propagated in the presence of blocking concentrations of MAb A6.1.2 or in 1/10 of the inhibitory concentration, suggesting that this mutation is stable in culture.

A second change of G to A at nucleotide 873 that translated to an E-to-K substitution at amino acid residue 296 was identified. The population sequenced contained a mixture of these two residues. A K at position 296 was reported in the sequence of the original MNV-1 isolate (22). Serial passage of MNV-1 in RAW 264.7 cells resulted in a mixture of K and E in the passaged population and then an E in the final passage (P3) (40). It is unlikely that this residue contributes to resistance to neutralization in this study because MAb A6.1.2 neutralizes both the original isolate (22) and the RAW 264.7-adapted CW.1 P3 clone (40).

**Predicted location of the MNV-1-neutralizing epitope on VP1.** MNV-1 and NV VP1 are 40.6% identical over the full-length of the protein (15), with the most significant variation being predictably present in the P2 domain (Fig. 4). NV is the only norovirus strain for which crystallographic data have been reported. Therefore, the sequence alignments shown in Fig. 4 were used to gain insight into a potential relationship between the peptide derived from phage display and the mutation in the neutralization-resistant viruses. Regions of the NV VP1 sequence that aligned with the relevant MNV-1 residues were identified and are highlighted on the VP1 structure (Fig. 4 and 5). The L386F mutation was in close proximity to W375-S378 of NV VP1 on the surface of the molecule. Residues G333, Q334, and L335 of the phage library-derived epitope aligned near G319-D327 of VP1 sequence. These two regions in the NV VP1 structure that corresponded to the escape mutant and phage peptide were in close proximity to each other, and a large portion is surface exposed.

**DISCUSSION**

Studies of viral attachment to host cells for the noncultivable human noroviruses have utilized several different approaches that include mutational analysis using self-assembling VLPs, isolated domains of VP1, and assays including epithelial cell binding, synthetic carbohydrate and saliva binding, and hemagglutination. We have used phage-displayed oligopeptide libraries to identify human norovirus epitopes reactive with MAb that block these interactions. Using MNV-1 as a model...
to further our understanding of norovirus-host cell interactions, we sought to define relationships between epitopes selected from the phage libraries, amino acid substitutions in VP1 generated in neutralization escape mutants, and domains of VP1 of human noroviruses important for putative receptor binding.

The peptide isolated from the phage library showed interesting similarities with the peptide selected by anti-SMV MAb 61.21 (24). The residues that could be aligned with MNV-1 VP1 are adjacent to the 61.21 epitope within a highly conserved consensus sequence, AP(L/I)GXPD, present in all norovirus strains sequenced to date, including MNV-1. The degree of sequence conservation N and C terminal to this peptide rapidly decreases. Despite the similarity in the sequence and position of the epitope, we have not observed cross-reactivity between MNV-1 and SMV antibodies (data not shown). These observations together support the previous assertion that this conserved sequence forms a structural landscape upon which variable residues coordinate to dictate antibody reactivity and putative receptor-binding specificity (24). Of note, the aspartic acid and histidine residues in the A6.2.1 epitope were highly conserved in the phage clones and were in spatially similar positions as the anti-NV MAb 54.6-selected epitope WTRGD HILH (24). However, the NV epitope location is speculative because of its lack of continuous residues that could definitively be aligned to VP1. Therefore, the relationship between the NV and MNV-1 peptides remains uncertain.

A leucine-to-phenylalanine change at position 386 was the only mutation associated with neutralization resistance. This location is 51 amino acids downstream from the epitope selected from the phage library. Both epitopes were defined with the same MAb, suggesting that their relationship lies in the tertiary structure of VP1. The structure of MNV-1 has not yet been solved, so epitope locations are proposed by projection onto the NV structure guided by sequence alignments. The site on the NV VP1 structure that aligned with L386 was close to the position of the C-terminal residues of the phage peptide projected onto the same structure. Together, these data suggest an important contribution of the amino acid residues defined here to neutralizing antibody recognition.

Regions of antibody complementarity to antigen have been found to be in close contact with 16 amino acid residues over 750 Å of solvent accessible surface (1, 6). The relationships between antibody-antigen surface area contact and amino acid proximity should thus be similar to the binding region of the MAb that neutralizes MNV-1, yet only the L386F substitution was identified. Mutated amino acids associated with resistance to neutralization do not necessarily disclose the entire antibody-antigen binding region; they identify only the residues in the epitope that are not constrained by the need to maintain structure required for biological activity. Residues in VP1 important for interactions with the MAb that were not identified by analyses of neutralization escape mutants must be required for the biological activity of the virus particle. Mutations in these regions would never be observed because these mutants would have been rendered inactive or noninfectious. Such mutation residue site restrictions are commonly observed in neutralization escape mutant analyses (3, 27, 30). The use of phage-displayed peptide libraries thus provides a complementary approach to understand epitopes involved in MNV-1 neutralization. This approach does not require infectivity for epitope analysis and thus is not subject to the same constraints. Amino acid residues that are reactive with MAb A6.2.1 defined by phage display could potentially reveal more of the biologically active region of the MAb recognition site.

Changes resulting in altered antigenic properties of viruses nearly always occur on the capsid surface and have been attributed to the substitution of residues directly involved in binding antibody, yet it is possible that neutralization resistance is conferred by amino acid substitutions distant from the antibody binding site (29). We cannot definitively conclude from escape mutant analysis alone that the site identified is directly responsible for virus attachment to cells. Mutations introduced at locations other than the antibody binding site can cause conformational perturbations in the capsid that alter the actual binding site, leading to neutralization resistance.
However, epitopes on VP1 of two norovirus strains that are important for interactions with cells localize to a similar position on the protein, and this observation increases the possibility that this region of VP1 is directly involved in cell attachment. Further evidence to suggest that the region defined by the SMV epitope, the MNV-1 peptide selected from the phage library, and the mutation in the MNV-1 escape mutant may directly mediate receptor binding is provided by a recent structural analysis of the P domain of human norovirus strain VA387 in complex with blood group trisaccharides (5). Amino acid residues predicted to form contact sites with carbohydrates are adjacent to the sites defined by us in this study (Fig. 4). In addition, a mutational analysis of the P domain revealed that threonine residue 338 of VA387, also within our predicted neutralization site, was associated with carbohydrate binding, and mutation of this residues abolished the interaction (36). The MNV-1 receptor has not yet been identified, but the data presented here suggest that although receptors for human norovirus and MNV-1 may be different, the functional domains on the norovirus particle important for interactions with cells may be similar.

A phage library screen and generation of neutralization escape mutants to investigate the A6.2.1 antibody recognition site of MNV-1 have identified the first amino acid residues of MNV-1 VP1 that contributes to antibody neutralization. These data also provide additional evidence that the regions identified by MAb A6.2.1 and other norovirus MAb are structurally important domains of the capsid protein that could be targets for the development of cell-binding inhibitors.

FIG. 5. Putative location of MNV-1-neutralizing epitope. Shown is the VP1 monomer illustrating the location of amino acid residues of NV that align with the MNV-1 peptide selected from the phage library (red) and the escape mutant (yellow). The residues in yellow are NV amino acids W375, S377, and P378. The residues in red are D327, W328, and H329. The structure was drawn and analyzed with NCBI CN3D4.1.

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