Noroviruses are major etiological agents of acute viral gastroenteritis worldwide and are estimated to be responsible for approximately 200,000 deaths in children under 5 years of age each year, mainly in the developing world (48). Noroviruses are often associated with outbreaks in closed settings, such as schools, hospitals, hotels, cruise ships, nursing homes, and military facilities, and they are considered the most common cause of nonbacterial gastroenteritis outbreaks (18).

Noroviruses, which belong to the family Caliciviridae, contain a single-stranded, positive-sense RNA genome that is organized into three open reading frames (ORFs 1 to 3). ORF1 encodes a polyprotein that is cotranslationally cleaved into the nonstructural proteins by the viral protease, ORF2 encodes the major capsid protein (VP1), and ORF3 encodes a small basic protein (VP2) that has been associated with stability of the capsid (6,18). The expression of VP1 results in the formation of virus-like particles (VLPs), which have been shown to be morphologically and antigenically similar to native virions (28). X-ray crystallographic analysis of the capsid revealed a $T=3$ icosahedral symmetry, with 180 VP1 molecules organized into 90 dimers. Each VP1 monomer is composed of two domains, the shell (S) and protruding (P) domains, linked by a flexible hinge. The S domain forms the internal icosahedral scaffold, from which the P domain projects to form arch-like structures (49). The P domain can be divided further into P1 and P2 subdomains, with P2 being the most exposed and variable region of VP1 (49). Recent X-ray analyses have shown that the P2 subdomain interacts with synthetic carbohydrates corresponding to various ABH histo-blood group antigens (HBGA) (9, 10). Interestingly, the expression of the S domain alone results in smooth particles with a diameter of approximately 30 nm, while expression of the P domain alone yields particles with a diameter of approximately 20 nm (6, 56). The P particles (which can be produced rapidly in bacterial and yeast expression systems) are antigenically similar to the P domain of intact VLPs and have been used in structure and function studies to map specific amino acid residues in the P2 domain that are involved in carbohydrate interactions (9, 56–58).

Noroviruses have been classified into six genogroups (GI to GVI) and multiple genotypes (44, 68) based on VP1 sequences. Noroviruses from GI, GII, and GIV have been shown to infect humans, with GII strains being the most prevalent worldwide (18). Since the mid-1990s, noroviruses from genotype GII.4 have been the most common cause of outbreaks of gastroenteritis in the United States and Europe (19, 53, 69). It has been reported that the chronological emergence of new variants (or phylogenetically related clusters) of GII.4 noroviruses correlated with an increase in the occurrence of large epidemics throughout the world (19, 39, 52, 53, 61, 69). In 2002, a GII.4 variant designated the Farmington Hills cluster spread rapidly in the human population and displaced the previous GII.4 strains (53, 61). The mechanisms that have driven the fitness of this variant have not been established completely, but a number of mutations in ORF1 and ORF2 have been detected and studied extensively. Interestingly, this strain presented a unique amino acid (aa) insertion (Gly394) in the P2 domain that was in close proximity to the HBGA binding site (9) and altered the binding of VLPs to synthetic carbohydrates (12, 39).

Studies of human noroviruses have been hampered by the lack of a cell culture system and small-animal disease models, making it difficult to assess the neutralization activity of antibodies. It has...
been reported that the measurement of antibodies that block the interaction between VLPs and HBGA carbohydrates might serve as a surrogate neutralization test (26, 40). Consistent with this, a correlation was found between the presence of serum HBGA-blocking antibodies and resistance to infection in adult volunteers challenged with Norwalk virus (NV) (50). Bok et al. (8) evaluated norovirus vaccine candidates in the chimpanzee model and found that chimpanzees vaccinated with Norwalk virus VLPs (GI.1), but not Hu/NoV/GII.4/MD145-12/1997/US (MD145-12) (GII.4) VLPs, were protected against reinfection with Norwalk virus. The protection from infection in the chimpanzee model after vaccination correlated with the presence of serum antibodies that blocked the binding of Norwalk VLPs to HBGA carbohydrates (8). Taken together, these data suggested that protective epitopes are located at or near HBGA binding sites on the virion.

Because VLPs are antigenically similar to native virions (28), efforts have been made to characterize the binding of monoclonal antibodies (MAbs) developed after immunization of mice with VLPs from various norovirus genotypes (3, 25, 66, 67). Several cross-reactive MAbs have been identified, and most of them have been mapped to the S domain or the C-terminal region of the P1 domain (3, 4, 35, 45, 46, 51, 65, 66). It has been reported that certain MAbs block the interaction of VLPs with cells or synthetic HBGA carbohydrates (38, 43), and two HBGA-blocking sites were recently mapped (11, 37).

In this study, we developed MAbs against the capsid protein of a GI.4 strain from the Farmington Hills cluster to explore the antigenic properties of this genetic cluster. Using competition assays and newly developed genogroup I/II chimeric VLPs, we were able to show that multiple epitopes on the norovirus capsid can be involved in the blocking of VLP-HBGA interactions. In addition, further analyses identified HBGA-blocking MAbs that mapped to a region of the capsid that has been associated with the emergence of new GI.4 variants.

**MATERIALS AND METHODS**

**Expression and purification of VLPs.** To express VLPs, the ORF2 and ORF3 genes of norovirus strain Hu/NoV/GII.4/MD2004-3/2004/US (MD2004-3) (29) were amplified by PCR and cloned into a pENTR plasmid (Invitrogen, Carlsbad, CA) to yield pENTRM2004-3. Recombination of plasmid DNA with baculovirus DNA was performed using a Baculolodekt o3 kit (Invitrogen), and a baculovirus stock was obtained following transfection of the recombination product into Sf9 cells (serum-free adapted Sf9 cells; Invitrogen) as recommended by the manufacturer. The baculovirus stock was used to infect Sf9 suspension cultures for VLP production. Culture medium from baculovirus-infected cells was layered onto a 25% (wt/vol) sucrose cushion and subjected to centrifugation in an SW28 rotor at 76,200 × g for 4 h at 4°C (7, 20). The resulting pellets were dissolved in phosphate-buffered saline (PBS), pH 7.4, and further purified through a cesium chloride (CsCl) gradient by centrifugation in an SW55 rotor at 218,400 × g for 18 h at 15°C. The collected fractions (densities of ~1.3 g/ml) were dialyzed against PBS, and the protein concentration was determined with a commercial Bradford assay kit (Pierce, Rockford, IL). The presence of VLPs was confirmed by electron microscopy. The expression and purification of VLPs from Hu/NoV/GI.1/Norwalk/1968/US, Hu/NoV/GI.3/DesertShield395/1990/US, Hu/NoV/GI.1/Hawaii/1971/US, Hu/NoV/GI.2/SnowMountain/1976/US, Hu/NoV/GI.3/Toronto24/1991/CA, Hu/NoV/GI.1/SaintCloud624/1998/US, Hu/NoV/GI.4/CHDC5191/1974/US, Hu/NoV/GI.4/CHDC4871/1977/US, Hu/NoV/GI.4/HS-191/2001/US, and Hu/NoV/GI.4/MD145-12/1997/US are described elsewhere (7, 14, 20, 21, 33, 34).

**Production of MAbs.** BALB/c mice were immunized subcutaneously with 100 μg of MD2004-3 VLPs four times in 2-week intervals. After determination of the serum titers, mice were boosted again intravenously with 100 μg of MD2004-3 VLPs. Three days later, mice were sacrificed, and spleen cells were isolated and fused with myeloma cells as described previously (31). The reactivity of the single-clone hybridoma supernatants was tested against MD2004-3 VLPs, and positive cells were collected for further characterization. Animal experiments and MAb production were carried out at Creative Biolabs (Shirley, NY) and conducted under approved protocols at Stony Brook University (IACUC permit 2010-1632). All efforts were made to minimize suffering. The isotype of each antibody was determined with an ICS-2 mouse antibody isotyping kit (Sigma, St. Louis, MO) following the manufacturer’s recommendations.

**ELISA.** The reactivity of each MAb against norovirus VLPs was examined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well polystyrene microtiter plates (Thermo, Milford, MA) were coated with 100 μl of purified VLPs at a concentration of 0.5 μg/ml in PBS, pH 7.4, and incubated overnight at 4°C. Wells incubated with PBS alone were used as a negative control for MAb binding. Wells were washed with PBS containing 0.1% Tween 20 (PBS-T) and blocked with PBS–5% fat-free milk for 1 h at room temperature (RT). Each MAb was used at 5 μg/ml and adsorbed for 2 h at RT. The binding of antibodies to the VLP antigen was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (1:2,000 dilution; KPL, Gaithersburg, MD) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; KPL). The binding of VLPs to the plate was confirmed with guinea pig hyperimmune sera (1:500 dilution) raised against each of the homologous VLPs, except for GI.2 VLPs, for which GI.1 hyperimmune serum was used.

**Western blot analyses.** The reactivity of each MAb with MD2004-3 VLPs was analyzed by Western blotting. Briefly, 2.5 μg of VLPs was mixed with Novex 2× Tris-glycine-SDS loading buffer (Invitrogen), boiled for 5 min at 95°C, and separated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electroblotted onto a nitrocellulose membrane using a iber blot dry blotting system (Invitrogen). The membranes were blocked with PBS–5% fat-free milk for 1 h at RT. Each MAb (1:1,000) was adsorbed for 2 h at RT, and binding was detected with HRP-conjugated goat anti-mouse immunoglobulin G (1:2,000 dilution; KPL) and the peroxidase substrate ABTS (KPL). A suspension of MD145-12 VLPs (1 μg/ml) and a norovirus-negative stool were used as controls.

**Blocking of binding of VLPs to synthetic HBGA by MAbs.** The MD2004-3 VLPs were screened for binding to a panel of HBGA-associated oligosaccharides as described elsewhere (8). The oligosaccharides examined in this study were type A, type B, Lea, Leb, Lec, and H type 1 (H1), H2, and H3 bovine serum albumin (BSA). A 10% stool suspension of MD145-12 was incubated for 2 h, and the binding of native proteins was determined by incubation with anti-MD145-12 VLP guinea pig hyperimmune serum (1:2,000 dilution), followed by incubation with peroxidase-conjugated goat anti-guinea pig immunoglobulin G (1:2,000 dilution; KPL) and the peroxidase substrate ABTS (KPL). A suspension of MD145-12 VLPs (1 μg/ml) and a norovirus-negative stool were used as controls.
VLPs that were not incubated with MAb or sera. A blocking value of \( \frac{1}{4} \) of binding was considered to be under the cutoff value (26, 40).

**Construction and expression of chimeric VP1.** The NV ORF2 sequence was PCR amplified from plasmid NV FL101 (15) by use of the specific primers NV-SalIf and NV-NotIr (Table 1), which introduced unique SalI and NotI restriction sites at the 5'- and 3'-ends, respectively. The PCR fragment was gel purified, digested with SalI and NotI, and ligated into the corresponding sites of a cut pCI expression vector, generating a plasmid designated pCI-NV (Fig. 1A). A QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was employed to mutagenize pCI-NV, using forward (NV-PspXIf) and reverse (NV-PspXIr) primers to introduce a PspXI restriction site at the hinge region (Fig. 1B and Table 1). This clone, designated pCI-NV-PspXI, served as an intermediate vector for swapping capsid domains.

To generate an NV S domain–MD2004-3 P domain chimera, the following steps were performed. With pENTRMD2004-3 as the template, primers MD2004-3-PspXIF and MD2004-3-NotIr were used to generate an amplicon of the MD2004-3 P domain with flanking PspXI and NotI restriction sites. The amplicon was digested with NotI and PspXI and ligated into the pCI-NV-PspXI backbone, which had been digested with the same restriction enzymes, thereby replacing the NV P domain with the MD2004-3 P domain. The chimeric ORF2 plasmid was designated pCI-NV/MD2004-3 (Fig. 1C). A similar approach was applied to obtain an MD2004-3 shell domain–NV P domain chimera. Using the same clone of the MD2004-3 VP1-VP2 sequence as a template, we used primers MD2004-3-SalIf and MD2004-3-PspXIR to generate an amplicon of the MD2004-3 shell domain with flanking SalI and PspXI restriction sites.

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**TABLE 1** Primers used for construction of GI.1/GII.4 chimeric capsid proteins

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>NV-PspXIf</td>
<td>gttttagttctctacgCgcaggagaagagacgag</td>
</tr>
<tr>
<td>NV-PspXIR</td>
<td>gcttgtttctcgcgcagtggagggctaataacac</td>
</tr>
<tr>
<td>NV-SalIf</td>
<td>CCGCTGGTACCTCTAGAG</td>
</tr>
<tr>
<td>NV-NotIr</td>
<td>CATGTCTGCTGAGCGGCC</td>
</tr>
<tr>
<td>MD2004-3-PspXIf</td>
<td>gttttagttctctacgCgcaggagaagagacgag</td>
</tr>
<tr>
<td>MD2004-3-NotIr</td>
<td>GTCTGGTACCTCTAGAG</td>
</tr>
<tr>
<td>MD2004-3-PspXIr</td>
<td>gttttagttctctacgCgcaggagaagagacgag</td>
</tr>
</tbody>
</table>

a Introduced mutations are shown with bold uppercase letters, NV sequences are shown in italics, MD2004-3 sequences are shown with bold lowercase letters, and plasmid sequences are shown with uppercase letters.

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**FIG 1** Construction of chimeric VP1 proteins. (A) Schematic diagram showing wild-type NV VP1. Amino acid numbers are in bold, and nucleotide numbers are italicized. The amino acid and nucleotide numbers corresponding to the borders of the shell (S) and protruding (P) domains are shown. The amino acid and nucleotide sequences of the hinge region are shown below. (B) A recombinant NV VP1 was engineered in which the valine at position 224 was replaced by leucine, generating a hinge region identical to that of the Hu/NoV/GII.3/Saga/5424/2003/JP strain (GenBank accession no. AB242256). The V224L substitution introduced a unique PspXI restriction site (the nucleotide sequence of the PspXI restriction site is underlined). (C and D) Chimeric VP1 expression vectors were generated by cloning either the P or S domain of the MD2004-3 strain into the pCI-NV-PspXI backbone vector.

The PCR fragment was gel purified, digested with SalI and NotI, and ligated into the corresponding sites of a cut pCI expression vector, generating a plasmid designated pCI-NV (Fig. 1A). A QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was employed to mutagenize pCI-NV, using forward (NV-PspXIf) and reverse (NV-PspXIr) primers to introduce a PspXI restriction site at the hinge region (Fig. 1B and Table 1). This clone, designated pCI-NV-PspXI, served as an intermediate vector for swapping capsid domains.

To generate an NV S domain–MD2004-3 P domain chimera, the following steps were performed. With pENTRMD2004-3 as the template, primers MD2004-3-PspXIF and MD2004-3-NotIr were used to generate an amplicon of the MD2004-3 P domain with flanking PspXI and NotI restriction sites. The amplicon was digested with NotI and PspXI and ligated into the pCI-NV-PspXI backbone, which had been digested with the same restriction enzymes, thereby replacing the NV P domain with the MD2004-3 P domain. The chimeric ORF2 plasmid was designated pCI-NV/MD2004-3 (Fig. 1C). A similar approach was applied to obtain an MD2004-3 shell domain–NV P domain chimera. Using the same clone of the MD2004-3 VP1-VP2 sequence as a template, we used primers MD2004-3-SalIf and MD2004-3-PspXIR to generate an amplicon of the MD2004-3 shell domain with flanking SalI and PspXI restriction sites.
The amplicon was digested with SalI and PspXI and ligated into the pCI-NV-PspXI backbone, which had been digested with the same restriction enzymes, thereby replacing the Norwalk virus shell domain with the MD2004-3 shell domain. The chimeric VP1 plasmid was designated pCI-MD2004-3/NV (Fig. 1D). All constructs were confirmed by nucleotide sequencing. The expression of chimeric VLPs was achieved as described above.

Site-directed mutagenesis for epitope mapping. The ORF2 sequence of MD2004-3 was amplified from pENTR MD2004-3 with primers MD2004-3-SalIF and MD2004-3-NotIR, digested with the corresponding enzymes, and ligated into a pCI vector. Site-directed mutagenesis of pCI-MD2004-3 was performed using a QuikChange site-directed mutagenesis kit (Stratagene) and complementary forward and reverse primers which carried the nucleotide mutations (primer sequences are available upon request). The restriction enzyme DpnI (10 U/µl) was used to digest the parental DNA. Each of the mutated products was transformed into E. coli XL1-Blue supercompetent cells (Stratagene). Transformed cells were grown overnight in LB plates with carbenicillin (50 µg/ml), and individual colonies were used for plasmid amplification. The resulting plasmids were subjected to sequence analysis to verify the entire VP1 coding region and confirm the presence of the introduced mutations. MD2004-3 mutant VLPs were expressed and purified as described above.

Immunofluorescence microscopy. HeLa cells were plated in 96-well plates at 50,000 cells/well and infected with a modified vaccinia virus expressing bacteriophage T7 RNA polymerase (MVA-T7) at a multiplicity of infection (MOI) of 5 PFU/cell for 1 h (62). After infection, cells were transfected with 400 ng/well of each DNA construct by use of Lipofectamine 2000 (Invitrogen) following the manufacturer’s recommendations. Cells were incubated for 24 h and then fixed with cold methanol for 20 min. The optimal dilution (1:200) of MAb was determined by serial dilution. Goat anti-mouse IgG(H+L) conjugated with Alexa Fluor 594 (Molecular Probes-Invitrogen, Carlsbad, CA) was used for detection. Guinea pig hyperimmune sera raised against MD2004-3 VLPs and goat anti-guinea pig IgG(H+L) conjugated with Alexa Fluor 594 (Molecular Probes-Invitrogen) were used to confirm expression of the antigens.

Bioinformatic analyses. Nucleotide sequences from the prototype strains were downloaded from GenBank (accession numbers are shown in Fig. 2) and aligned by using the translated aa sequences. Phylogenetic trees were constructed using the Kimura two-parameter model as a nucleotide substitution model and a neighbor-joining (NJ) algorithm as implemented in MEGA v4.0 (55).

The solved structure of the P domain of VA387 virus (GII.4) in complex with carbohydrate (Protein Data Bank [PDB] accession number 2OBT) was used to identify the residues involved in binding with MAbs and was visualized by using MacPyMol (DeLano Scientific LLC).

Competition assays. For competition assays, MAbs were biotinylated by using an EZ-Link sulfo-NHS-LC biotinylation kit (Thermo Scientific, Rockford, IL) following the manufacturer’s recommendations. Ninety-

FIG 2 Phylogenetic tree of noroviruses showing the different genogroups and GI.4 strains (shaded box). The genogroup is indicated to the right of each phylogenetic cluster. The MD2004-3 GI.4 norovirus that was used to generate VLPs and monoclonal antibodies in this study is marked by a filled square. Noroviruses serving as the source for additional VLPs analyzed in this study are indicated with filled circles.
reactive MAb concentrations, based on the value of the PBS control (i.e., the percent competition or enhanced binding was determined for all competitive reactions). The optimal concentration of each biotin-conjugated MAb was determined to yield an optical density at 450 nm (OD450) of 0.8 to 1.5 by binding with MD2004-3 VLP-coated plates. The percent competition or enhanced binding was determined for all competitor MAb concentrations, based on the value of the PBS control (i.e., the value of binding of biotin-conjugated MAb to MD2004-3 VLPs).

Blocking of MD2004-3 hyperimmune serum by MAbs. The blocking activity of each MAb or pool of MAbs against the binding of polyclonal hyperimmune serum directed against MD2004-3 VLPs was examined by ELISA. Briefly, 96-well polyvinyl microtiter plates (Thermo) were coated with 100 μl (125 ng/ml) of MD2004-3 VLPs and incubated overnight at 4°C. Wells were washed with PBS-T and blocked with PBS-T with 1% BSA for 1 h at RT. Serial dilutions of each competitor MAb were made, with a starting concentration of 10 μg/ml, and were absorbed for 2 h at RT. Each biotin-conjugated MAb was absorbed for 2 h at RT, and binding to the VLP antigen was detected with high-sensitivity streptavidin-horseradish peroxidase (1:10,000 dilution; Thermo Scientific) and ABTS (KPL). The concentration of MD2004-3 VLPs used for coating was optimized in homologous competitions. The optimal concentration of each biotin-conjugated MAb was determined to yield an optical density at 450 nm (OD450) of 0.8 to 1.5 by binding with MD2004-3 VLP-coated plates. The percent competition or enhanced binding was determined for all competitor MAb concentrations, based on the value of the PBS control (i.e., the value of binding of biotin-conjugated MAb to MD2004-3 VLPs).

Blocking of MD2004-3 hyperimmune serum by MAbs. The blocking activity of each MAb or pool of MAbs against the binding of polyclonal hyperimmune serum directed against MD2004-3 VLPs was examined by ELISA. Briefly, 96-well polyvinyl microtiter plates (Thermo) were coated with 250 ng/ml of MD2004-3 VLPs and incubated overnight at 4°C. Wells were washed with PBS-T and blocked with PBS-T with 1% BSA for 1 h at RT. Each MAb (10 μg/ml), singly or in pooled solutions, was absorbed for 2 h at RT. Wells were washed with PBS-T, and 10-fold serial dilutions of an MD2004-3 hyperimmune serum were incubated for 1 h at RT. The binding of hyperimmune serum to the VLP antigen was detected with HRP-conjugated goat anti-guinea pig immunoglobulin G (1:2,000) and ABTS (KPL).

RESULTS

Expression and purification of MD2004-3 VLPs. Strain MD2004-3 was identified in 2004 as a causative agent of diarrhea in Baltimore, MD, hospital outbreak (29). This strain grouped within the Farmington Hills cluster of the GI.4 genotype (Fig. 2) that emerged in 2002 and became the globally predominant GI.4 strain during 2002 and 2004 (53, 61). In order to develop VLPs for further study, the ORF2 (VP1) and ORF3 (VP2) genes of the MD2004-3 strain were engineered into a recombinant baculovirus, and the norovirus capsid proteins were expressed in baculovirus-infected insect cells. Following concentration of the resulting VLPs by ultracentrifugation in CsCl gradients, visible bands were collected and dialyzed against PBS. Denaturing polyacrylamide gel electrophoresis of the proteins recovered from the CsCl gradient showed two bands that migrated between 50 and 64 kDa (Fig. 3A, lane 2). The presence of VLPs was confirmed by electron microscopy (Fig. 3B).

Isolation of MD2004-3-specific MAbs and their reactivity with other VLPs. After two fusions, 16 hybridoma clones were screened as positive for reactivity with MD2004-3 VLPs. Isotyping identified 10 IgM-secreting and 6 IgG-secreting hybridomas: this study focused on characterization of the IgG MAbs. The IgG MAbs were further isotyped as subclass IgG1 (A10, B11, and B12), IgG2a (A3 and B15), and IgG2a (A6) MAbs (Table 2). All six MAbs reacted with high titers (>10⁴) against the MD2004-3 VLPs by ELISA, but none was reactive by Western blotting, suggesting that they each recognized conformational epitopes (Table 2). The reactivity of each MAb was further evaluated by ELISA, using a panel of 10 additional VLPs available in our laboratory (Fig. 4). Four distinct patterns of cross-reactivity could be seen with other GII VLPs (Fig. 4). The first pattern was represented by MAb B15, which showed cross-reactivity among all GII.4 VLPs in the panel and low cross-reactivity with a GII.3 strain. The second pattern, exhibited by MAbs A10, B11, and B12, showed strong reactivity and specificity for the immunizing GII virus, MD2004-3, only. A third pattern, represented by MAb A6, showed strong reactivity with MD2004-3 and weak reactivity with MD145-12. Finally, MAb A3 was strongly reactive with GII.4 strains detected in 1987 and 2004 (i.e., MD145-12, HS-191, and MD2004-3) and, to a lesser extent, with GII.4 VLPs from 1974 and with GII.1 and GII.2 viruses. To determine whether the MAbs described here could recognize native GII.4 norovirus virions, biotinylated MAbs were attached to NeutriAvidin-coated plates and tested against 10% stool suspensions positive for MD145-12 virus. As expected, the three MAbs (A3, A6, and B15) that reacted with MD145-12 VLPs were also able to detect native viral proteins present in stool sam-

![FIG 3 Expression and antigenic characterization of wild-type and chimeric VLPs. (A) SDS-PAGE showing the masses of the expressed proteins. VLPs were analyzed by Tris-glycine SDS-PAGE in a 10% gel stained with Coomassie blue. (B) Electron micrographs of VLPs expressed in the baculovirus system and visualized by negative staining with 3% phosphotungstic acid, pH 7.2. (C) Reactivity of MAbs with chimeric VLPs. Binding of MAbs was measured by ELISA as described in Materials and Methods. The black shaded squares represent positive binding, and white squares represent no binding (see Fig. 4 legend for positive and negative cutoff values). The nomenclature of each VLP is described in Materials and Methods.](http://jvi.asm.org/7418/7750/Fig3.jpg)
FIG 5 Detection of native MD145-12 proteins in stool samples. Biotinylated MAbs were incubated in NeutrAvidin-coated plates, and a 10% stool suspension of MD145-12 was incubated for 2 h. Binding of captured native proteins was determined by incubation with anti-MD145-12 VLP hyperimmune serum, followed by incubation with a peroxidase-conjugated goat anti-guinea pig immunoglobulin G and the peroxidase substrate ABTS. A suspension of MD145-12 VLPs (1 μg/ml) and a norovirus-negative stool were used as controls. The experiment was performed twice in duplicate wells. Bars represent the means, the error bars represent the standard errors of the means (SEM), and the dashed line represents the limit of detection.

FIG 4 Reactivity of MAbs raised against MD2004-3 virus with a panel of VLPs. Binding of MAbs (labeled for each VLP experiment as indicated in the bottom panels) was measured by ELISA as described in Materials and Methods. Reactivity of VLPs with hyperimmune sera is represented by black bars. Student’s t test was used to compare reactivity differences of weakly positive MAbs and the negative control. Statistics were assessed using GraphPad Prism 5.0. Statistical significance is denoted by asterisks: *, P ≤ 0.05; **, P ≤ 0.001. The dashed line represents the cutoff value.

Multiple Epitopes Involved in Norovirus HBGA Blocking
the VLP-carbohydrate interaction occurred in a dose-dependent manner (Fig. 6B).

Identification of binding domain (S or P) specificity in VP1. The capsid protein (VP1) of noroviruses is composed of two domains, S and P. In order to map the domain of VP1 recognized by the GII.4 MAbs, a strategy was developed to generate chimeric VP1 proteins with S and P domain swaps between MD2004-3 (pCI-MD2004-3) and Norwalk virus (pCI-NV) were constructed, and the expression of VP1 was verified with the corresponding hyperimmune serum by immunofluorescence (data not shown). A unique PspXI restriction endonuclease site was introduced between the S and P coding sequences in the Norwalk virus clone (yielding pCI-NV-PspXI) to allow the exchange of regions between Norwalk virus and MD2004-3. The resulting plasmids, carrying chimeric VP1 genes, and the control plasmids pCI-MD2004-3 and pCI-NV were transfected into HeLa cells in the presence of MVA-T7 to increase expression efficiency (17, 62), and the binding of monoclonal antibodies was examined by immunofluorescence. All of the MAbs reacted with cells transfected with pCI-MD2004-3 but not with pCI-NV or empty pCI vector (Fig. 7). MAbs A3, A6, A10, B11, and B12 reacted with the chimeric VP1 protein that carried the P domain from MD2004-3, while MA B15 reacted with the chimeric VP1 protein that carried the S domain from MD2004-3 (Fig. 7).

To examine whether the conformational epitopes recognized by the MAbs in the eukaryotic expression studies would be present on intact VLPs, the chimeric VP1 genes were cloned and expressed using a baculovirus system as described in Materials and Methods. SDS-PAGE showed the presence of proteins that migrated the same distance as proteins with sizes between 50 and 64 kDa (Fig. 3A, lanes 3 and 4). The presence of self-assembled VLPs was detected by electron microscopy (Fig. 3B), and their chimeric antigenic profile was confirmed by Western blotting with GI- and GII-specific antisera (data not shown). The specificity of MA b binding to the purified VLPs in an ELISA was identical to that observed with the pCI-based constructs in transfected cells (Fig. 3C).

Fine mapping of putative epitopes. An alignment of the aa sequences of the four VP1 proteins corresponding to the GII.4 VLPs used in this study showed the presence of 31 variable sites in the P domain that might be involved in epitope specificity (data not shown). To directly map residues involved in the formation of the various epitopes, we examined the predicted locations of the aa residues on the mono- and dimeric models of the crystal structure of the VP1 protein, including exposure on the surface of the capsid and proximity to HBGA binding sites. We targeted 14 aa residues as possible determinants of antigenic specificity (Fig. 8), and the pCI-MD2004-3 DNA construct was subjected to site-directed mutagenesis (Fig. 8A). Even though residues 411 and 412 were not in close proximity to the HBGA binding sites, mutagenesis of these residues was performed because they were identical in the MD2004-3, HS-191, and MD145-12 strains and represented a potential site for the cross-reactive MAb A3 or A6. The resulting constructs were transfected into HeLa cells, and binding of each of the MAbs was examined by immunofluorescence assay. One construct carrying the AD294GI mutations lost reactivity with MAbs A6 and A10 (Fig. 8A). To confirm this finding, we cloned the VP1 gene encoding the AD294GI mutations into a baculovirus vector. MD2004-3 mutant VLPs were used to confirm the loss of reactivity with MAbs A6 and A10 (Fig. 8A). To confirm this finding, we cloned the VP1 gene encoding the AD294GI mutations into a baculovirus vector. MD2004-3 mutant VLPs were used to confirm the loss of reactivity with MAbs A6 and A10 (Fig. 4). Analysis of the X-ray structure of VA387 revealed that residues 294 and 295 (indicated in red) are located in a loop between β-sheet 2 (β2) and β3, at the top of the P domain, at equal distances between the two HBGA binding sites (Fig. 8C)(9). Note that all other VLPs that tested negative by ELISA against MAbs A6 and A10 (Fig. 4) differed in sequence at aa residues 294 and 295 of the capsid protein (Table 3), providing further evidence of the importance of these aa resi-

FIG 6 MAbs block the binding of MD2004-3 VLPs to HBGA. (A) HBGA binding pattern of MD2004-3 VLPs. The ability of VLPs to bind to synthetic biotinylated carbohydrates was measured by ELISA. The graph represents the results from two experiments performed in duplicate, with each dot representing the mean for duplicate wells. (B) Percent binding of MD2004-3 VLPs to carbohydrate B in the presence of MAbs (starting concentration, 15 μg/ml) was calculated as described in Materials and Methods. Error bars represent SEM. The dashed line represents the 50% blocking cutoff value.
dues in the binding of MAbs A6 and A10. The binding of three P region MAbs (A3, B11, and B12) was not affected by the above mutations introduced into MD2004-3 VP1 (Fig. 8A and B).

Identification of antigenic sites. We next performed competition assays with biotinylated MAbs to define the number of antigenic sites in the capsid protein recognized by the MAbs in our panel. Note that unlabeled MAbs inhibited the binding of their biotinylated counterparts, in a dose-dependent manner. Representative results for the A3 MAb (competed only against itself) and the A6 MAb (competed against several MAbs) are shown in Fig. 9A. Four different antigenic areas could be identified based on cross-competition assays (Fig. 9B). MAbs A3 and B15 showed homologous competition only, suggesting that they bind to unique sites, designated antigenic sites I and III, respectively (Table 4). In addition, competition analyses of MAbs A6, A10, B11, and B12 suggested that they map in two overlapping antigenic sites, designated antigenic sites IIa (A6 and A10) and IIb (B11 and B12) (Table 4). The ability to block the binding of GIL4-specific hyperimmune serum to MD2004-3 VLPs by each MAb or combination of MAbs was examined by ELISA. A pool of six MAbs was able to block hyperimmune serum binding in a dose-dependent manner, with the maximum blocking obtained at dilutions of the hyperimmune serum between $10^{-3}$ and $10^{-4}$ (Fig. 10A). All of the MAbs that mapped in the P domain of the capsid were able to block the binding of hyperimmune serum to MD2004-3 VLPs, whereas MAb B15 (which mapped in the S domain) was not (Fig. 10B). Interestingly, when pools of three MAbs that represented at least one MAb from each of the antigenic sites were used, the blocking titers were similar to those of the pool of six MAbs. Moreover, no statistically significant difference was found in the blocking titer of MAb B11 or B12 compared to that of the pool of MAbs, suggesting that B11 and B12 represent an immunodominant epitope.

DISCUSSION

Despite the role of noroviruses as major etiological agents of acute viral gastroenteritis, research on vaccines has historically been hampered by the lack of a permissive cell line or robust animal model (18). Currently, recombinant VLPs serve as a tool to study the antigenic structure of noroviruses (23, 25, 28, 40, 54) and are in development as vaccine candidates (13, 27, 47, 60). Yet there are many unanswered questions regarding the effective design and development of norovirus vaccines, such as the identification of
FIG 8. Fine mapping of epitopes from MD2004-3 Mabs. (A) Immunofluorescence staining results for HeLa cells transfected with different DNA constructs. Mutations in VP1 from MD2004-3 (pCI-MD2004-3; WT) were introduced by using specific primers and a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s recommendations. (B) Electron micrographs and MAb reactivities of VLPs carrying AD294GI mutations. Binding of MAbs was measured by ELISA as described in Materials and Methods. (C) Structure of the P domain. Mutations in the structure of the P domain are colored light blue. Amino acids involved in the binding of Mabs A6 and A10 are shown in red. The HBGA binding sites are colored in pink, and the carbohydrate is shown in green.
antigenic sites involved in virus neutralization and protection from disease.

Over the last decade, GII.4 noroviruses have been the most common cause of gastroenteritis outbreaks in the United States and Europe (19,53, 69). In 2002, the Farmington Hills cluster of GII.4 viruses displaced older variants of GII.4 globally and became an important cluster from an evolutionary and epidemiological perspective (39,61). In order to further elucidate the antigenic properties of this genetic cluster, we developed and characterized a panel of six IgG MAbs against the capsid protein of MD2004-3, a Farmington Hills-like strain.

In concordance with evidence that norovirus genogroups present major antigenic differences (20, 59, 63), none of the MAbs detected VLPs from genogroups GI and GIV. Two (A3 and B15) of the six MAbs were cross-reactive with different GII VLPs, and the remaining four MAbs reacted with one (A10, B11, and B12) or two (A6) GII.4 VLPs. Interestingly, the epitopes of five of the MAbs mapped within the P domain, and that of one (B15) mapped within the S domain. Several cross-reactive MAbs have been described for noroviruses, and epitopes for most of them have been mapped within the most conserved regions of the norovirus capsid protein (C-terminal P or S domain). As suggested previously, cross-reactive MAbs could potentially be used in diagnostic assays (3,24, 36, 46, 51); however, Oliver et al. (45) described a cross-reactive MAb that recognized a linear epitope in the S domain of the VLPs from bovine noroviruses that did not detect native virions in fecal samples from experimental animals. They proposed that because the S domain forms the innermost domain of the capsid, the epitope is not accessible in native virions. In the 5-fold axis of the GII.4 capsid, there is a small surface-exposed region of the S domain that is conserved among GII strains (data not shown). Although it is possible that B15 might recognize this exposed region, it showed low reactivity with native MD145-12 virions in a 10% stool suspension. Together, these results suggest that certain S domain epitopes may not be optimal for detection of small quantities of viruses in stool, even though they are shown to be highly cross-reactive with purified VLPs.

The blocking of norovirus HBGA binding sites by sera from immunized animals or infected humans has been used as a surrogate for a norovirus neutralization assay (26, 40). Importantly, it was recently shown that the ability of sera to block VLP-HBGA interaction correlates with protection against infection in NV-vaccinated chimpanzees and against illness in infected human volunteers (8, 50). Five of the six MAbs described here could block the interaction of MD2004-3 VLPs with two synthetic HBGA carbohydrates (H3 and B), and as expected, their recognition epitopes mapped within the P domain. The S domain-specific MAb B15 did not block the interaction of MD2004-3 VLPs with

<table>
<thead>
<tr>
<th>Virus (genotype/strain/year)</th>
<th>aa at position(^a)</th>
<th>MAb reactivity(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GII.4/MD2004-3/2004</td>
<td>A D</td>
<td>++ +</td>
</tr>
<tr>
<td>GII.4/HS-191/2001</td>
<td>A G</td>
<td>− −</td>
</tr>
<tr>
<td>GII.4/MD145-12/1987</td>
<td>V G</td>
<td>+ −</td>
</tr>
<tr>
<td>GII.4/CHDC4871/1977</td>
<td>G I</td>
<td>− −</td>
</tr>
<tr>
<td>GII.4/CHDC5191/1974</td>
<td>G I</td>
<td>− −</td>
</tr>
<tr>
<td>GII.3/Toronto24/1991</td>
<td>T S</td>
<td>− −</td>
</tr>
<tr>
<td>GII.2/SnowMountain/1976</td>
<td>L Q</td>
<td>− −</td>
</tr>
<tr>
<td>GII.1/Hawaii/1971</td>
<td>V P</td>
<td>− −</td>
</tr>
</tbody>
</table>

\(^a\) Residues present in wild-type viruses. For the GenBank accession number of each strain, refer to Fig. 2.

\(^b\) ++, strong reactivity; +, weak reactivity; −, no reactivity (see Fig. 4).
carbohydrate H3, consistent with the likely internal location of its epitope in the capsid. Our unexpected finding that MAb B15 caused partial blocking of MD2004-3 VLPs with carbohydrate B (and not H3) suggests that binding of some antibodies in the S domain might also influence capsid interactions with certain carbohydrates.

Recently, two binding sites for MAbs developed against VLPs from different GII.4 strains were described. One site comprised a cluster of seven residues (i.e., residues 294 to 298, 368, and 372) (11), while the other included three residues (i.e., residues 407, 412, and 413) (37). Importantly, these residues were shown to interact with HBGA-blocking MAbs, suggesting that they may be associated with protective immunity (50). We used the various reactivity patterns of our panel of GII.4 MAbs against the VLPs to identify putative residues involved in determining their specificity and designed MD2004-3 capsids carrying mutations in those residues. Allen et al. (2) reported that after introduction of point mutations into recombinant GII.4 capsids, they failed to obtain recombinant VLPs for one of the four designed constructs, suggesting that the introduction of certain mutations can be structurally unfavorable for assembly. We showed that several mutant capsids engineered in this study reacted with the conformational MAbs and therefore acquired the correct folding. However, two of the MAbs (A6 and A10) in our study lost their reactivity when residues Ala 294 and Asp 295 were mutated to Gly and Ile, respectively, confirming that residues 294 and 295 are involved in the formation of an important antigenic site (1, 2, 11). Thus, these data are consistent with the involvement of this epitope in diversification of GII.4 strains and in immune responses against norovirus infection.

The identification and characterization of viral B cell epitopes have been important in establishing the roles of different antigenic sites in protection (5, 16, 32, 64). Using overlapping fragments of the norovirus capsid protein, Yoda et al. (67) have shown the presence of three continuous antigenic sites for a GII.3 strain (NV36 virus), with two in the S domain and one in the P domain. Because synthetic short peptides or overlapping fragments of the VP1 protein generally cannot be used to map discontinuous epitopes, competitive ELISAs have been employed widely in the topological mapping of MAbs that recognize conformational epitopes. Thus, using competitive ELISAs, Hale et al. (22) detected the presence of five antigenic sites in the capsid from GI/GII chimeric VP1 proteins, we identified the binding domains of the four antigenic sites. Specifically, one unique antigenic site (site I) and two overlapping sites (sites IIa and IIb) were detected in the P domain, while site III was mapped in the S domain. MAbs A6 and A10 share the same antigenic site (IIa), consistent with their loss of reactivity when residues 294 and 295 from the capsid were subjected to mutagenesis. MAbs B11 and B12 recognize overlapping epitopes in the same antigenic site (IIb), although competition assays and reactivity

<table>
<thead>
<tr>
<th>Antigenic site</th>
<th>Associated MAb(s)</th>
<th>Binding domain</th>
<th>HBGA-blocking activity</th>
<th>GII.4 VLPs that react with associated MAb(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A3</td>
<td>P</td>
<td>+</td>
<td>MD2004-3, HS-191, MD145-12, CHDC4871, CHDC5191</td>
</tr>
<tr>
<td>IIa</td>
<td>A6, A10</td>
<td>P</td>
<td>+</td>
<td>MD2004-3, MD145-12</td>
</tr>
<tr>
<td>IIb</td>
<td>B11, B12</td>
<td>P</td>
<td>+</td>
<td>MD2004-3</td>
</tr>
<tr>
<td>III</td>
<td>B15</td>
<td>S</td>
<td>−</td>
<td>MD2004-3, HS-191, MD145-12, CHDC4871, CHDC5191</td>
</tr>
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![FIG 10] MAb s block the binding of MD2004-3 hyperimmune serum to homologous VLPs. (A) Blocking activities of a pool of the six MAbs described in this study against different dilutions of guinea pig hyperimmune sera raised against MD2004-3 VLPs. (B) Reactivity of hyperimmune serum (dilution of 10⁻³) against MD2004-3 VLPs in the presence or absence of MAbs. Reactivity of hyperimmune serum with VLPs was measured as described in Materials and Methods. Each dot represents the mean for duplicate wells from a single experiment. Bars represent the average OD. Student’s t test was used to compare differences in blocking assay results and unblocked-control results. Statistics were assessed using GraphPad Prism 5.0. Statistical significance is denoted by asterisks: *, P ≤ 0.05; **, P ≤ 0.001.
patterns with different VLPs suggest that these MAbs map to different residues.

The ability of the pool of six MAbs to efficiently block the binding of a high-titer hyperimmune serum raised against GI.4 VLPs suggests that the major antigenic sites of the capsid are represented in the MAb panel. Interestingly, when combinations of three MAbs representing each of the three antigenic sites described for the P domain were examined, the blocking effect was similar to that of the pool of six MAbs. Thus, these data suggest that the three antigenic sites described here are likely immunodominant sites of the GI.4 norovirus capsid. It will be important to examine whether serum antibodies from immune individuals block the binding of these MAbs. If so, epitope blocking assays employing these MAbs might serve as a surrogate for virus neutralization.

Three possible mechanisms can be involved in the blocking of the HBGA binding site: (i) direct blocking, (ii) steric interference, or (iii) conformational changes that affect the structure. The fine mapping of MAbs A6 and A10 in conjunction with the fact that the other conformational MAbs, which mapped to different antigenic areas, did not significantly lose their reactivity after incubation with competitor MAbs (i.e., A3 and B15) provides evidence that steric interference is the mechanism by which these MAbs block the HBGA binding site of GI.4 noroviruses. Importantly, MAbs directed against the P domain have been shown to neutralize murine norovirus (MNV) infection (30, 42), and one MNV MAb has been shown to neutralize infection by covering the outer surface of the P domain without causing any apparent conformational changes in the capsid protein (30). Thus, because our GI.4 MAbs bind to multiple epitopes of the P domain and block HBGA-VLP interactions, they may prove useful in defining functional antigenic sites of human noroviruses in structural studies.

Norovirus vaccines based on VLP formulations have gained special interest because they have been shown to induce both systemic and mucosal immune responses when administered intranasally or orally (27). Recently, El-Kamary et al. reported data from phase I clinical trials in which NV VLPs were administered intranasally to humans, with monophosphoryl lipid A (MPL) and aluminum gel as adjuvants (13). No vaccine-related serious adverse events were reported, and high IgG and IgA titers developed when 100 µg of VLPs was administered. In addition, Bok et al. (8) have shown that chimpanzees vaccinated intramuscularly with GI VLPs, but not GII VLPs, were protected against infection with GI Norwalk virus, confirming the idea that multivalent vaccines may be required for protection against currently circulating noroviruses. In this regard, the S/P (S domain from one genogroup and P domain from another) chimeric norovirus VLPs might present the advantage of inducing broadly reactive immune responses and thus may constitute a new platform for norovirus VLP-based vaccines. It has been shown that the CD4+ epitopes are located in different domains of VLPs, depending on the norovirus strain (41). Thus, even though it is believed that most B cell neutralizing epitopes might be located in the P domain of the capsid, the presence of T cell epitopes in the S domain suggests that vaccination with S/P chimeric VLPs might enhance the immune response and cross-protection against different genogroups.

In summary, we report the generation and characterization of a new panel of MAbs directed toward epitopes on the capsid of GI.4 norovirus, the predominant norovirus genotype. The VP1 domain specificity of each MAb was established with a panel of VLPs and GI/GII chimeric VLPs, and the ability to block HBGA binding corresponded to specificity for the P domain. Modeling and mutagenesis studies established that evolution in the norovirus capsid protein may affect both antigenic and carbohydrate recognition phenotypes, consistent with a link between HBGA blocking and the potential neutralization activity of antibodies. Further characterization of the reagents developed in this study may facilitate the establishment of surrogates of protection against norovirus illness and may provide new approaches to development of candidate vaccines.

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