Epitopes in the P2 domain of norovirus VP1 recognized by monoclonal antibodies that block cell interactions

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Noroviruses cause the majority of epidemic outbreaks of acute viral gastroenteritis worldwide. Human norovirus strains do not grow in cell culture, but recent carbohydrate binding, sequence and structural analyses have begun to define functional domains in the norovirus capsid that may be conserved among multiple antigenic types. The purpose of this study was to localize domains and define sequences in the major capsid protein VP1 that are important for cell interactions. Monoclonal antibodies to genogroups GI.1 and GII.2 reference strains Norwalk virus and Snow Mountain virus, respectively, were generated that blocked binding of recombinant virus-like particles to Caco-2 intestinal cells and inhibited haemagglutination. Peptides that mimicked the mAb binding epitopes were selected from a phage-displayed random nonapeptide library. Anti-recombinant Norwalk virus mAb 54.6 and anti-recombinant Snow Mountain virus mAb 61.21 recognized epitopes located in the protruding P2 domain of VP1. The epitope recognized by mAb 61.21 contained amino acids that are completely conserved among norovirus strains across genogroups, including strains isolated from swine, bovine and murine species. This study identifies the first epitope involved in inhibition of norovirus–cell interactions and supports increasing evidence that interactions between noroviruses and host cells rely on structures in the P2 domain of VP1.

INTRODUCTION

Noroviruses are the major cause of epidemic food and waterborne outbreaks of acute viral gastroenteritis worldwide (Fankhauser et al., 2002; Glass et al., 2000; Koopmans et al., 2000; Mead et al., 1999). Infection rates are high and rapid person-to-person spread is common, resulting in large outbreaks that often persist. As outbreak surveillance and sequence analyses of norovirus strains isolated from epidemic and sporadic cases have increased over the past several years, the magnitude of genetic and likely antigenic diversity of these emerging pathogens is evident (Ando et al., 2000; Fankhauser et al., 1998, 2002; Green et al., 2000a; Lopman et al., 2004; Noel et al., 1999; Vinje et al., 2000). Norovirus strains that infect humans are classified into two major genogroups, GI and GII (Green et al., 2000b), and a tentative GIV (Vinje & Koopmans, 2000). Genogroups III and V contain bovine and murine norovirus strains, respectively (Han et al., 2004; Karst et al., 2003). GI and GII are further subdivided into 14 subgroups, GI.1–GI.7 and GII.1–GII.7 (Chakravarty et al., 2005). The genetic diversity among noroviruses in the major capsid protein VP1 suggests extensive antigenic diversity, but there are few data on how variability in VP1 defined by sequence analysis translates to variability in antigenic reactivity. Thus, it is important to determine which domains of norovirus capsids function in cell binding and which sequences in these domains may evoke neutralizing antibodies and a consequent protective immune response. The correlates of protective immunity are not known, but there is evidence that, if immunity to norovirus infection is induced, it is short-lived, suggesting that methods of passive immunotherapy or delivery of small molecule inhibitors may prove useful in outbreak control.

Study of norovirus biology is complicated by the inability to grow virus in laboratory cell-culture systems. However, the ability to purify virus-like particles (VLPs) from insect cells infected with recombinant baculoviruses that express VP1 and the minor capsid protein VP2 has led to significant discoveries related to norovirus structure and virus–host cell interactions (Bertolotti-Ciarlet et al., 2002; Jiang et al., 1992; Prasad et al., 1999; Tan et al., 2003; White et al., 1996). Norovirus capsids are composed of 180 copies of VP1 arranged to form a $T=3$ icosahedral virion (Prasad et al., 1994). VP1 folds into two major domains designated S for the shell domain and P for the protruding domain (Prasad et al., 1999). The S domain consists of the N-terminal 225 aa and contains elements essential for formation of the icosahedron (Bertolotti-Ciarlet et al., 2002). The P domain (aa

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226–330, Norwalk strain numbering) is divided into two subdomains, P1 and P2. The P domains interact in dimeric contacts that increase the stability of the capsid and form the protrusions on the virion seen in cryo-electron microscopic reconstructions (Prasad et al., 1994). The P2 domain is a 127 aa insertion (aa 279–405) in the P1 domain and is located at the most distal surface of the folded monomer. The P2 domain is the least conserved region of VP1 among norovirus strains, and the hypervariable region within P2 is thought to play an important role in receptor binding and immune reactivity (Kitamoto et al., 2002; Prasad et al., 1999; Tan et al., 2003; White et al., 1996).

Norovirus infection is associated with ABO blood type (Harrington et al., 2002; Hutson et al., 2002, 2004) and VLPs representative of both major genogroups GI and GII bind histo-blood group antigens on gastroduodenal epithelial cells and in saliva of individuals with secretor phenotypes (Harrington et al., 2002; Hutson et al., 2002, 2003; Jiang et al., 2004; Lindesmith et al., 2003; Marionneau et al., 2002; Tan et al., 2003). Norovirus-like particles representative of the major genogroups display distinct carbohydrate binding patterns (Huang et al., 2003), and these binding specificities may correlate with susceptibility and resistance to infection with different virus strains (Lindesmith et al., 2003). The relationship between norovirus infection and carbohydrate recognition in vitro was demonstrated by data showing that convalescent sera from infected volunteers blocked VLP binding to secreted carbohydrate in saliva samples (Harrington et al., 2002) and inhibited haemagglutination (Hutson et al., 2003).

To refine our understanding of the domains in VP1 and assembled capsids that are important for interactions with cellular receptors, we generated mAbs to recombinant Norwalk virus (rNV) VLPs and recombinant Snow Mountain virus (rSMV) VLPs. NV is the reference GI.1 strain and SMV is the reference GII.2 strain. One anti-rNV mAb blocked VLP binding to differentiated Caco-2 cells and inhibited haemagglutination. One anti-rSMV mAb also blocked haemagglutination in a specific and dose-dependent manner. Peptide mimics of the epitopes to which these mAbs were directed were identified by screening a phage-displayed random nonapeptide library. Both epitopes were conformational and mapped in the P2 domain near a putative binding pocket recently described by Tan et al. (2003). This study defines the first epitope on norovirus capsids recognized by blocking mAbs, provides strong evidence that the P2 domain contains critical determinants of interactions between noroviruses and host cells, and suggests a potential target for design of small molecule inhibitors that could prevent initial virus–host cell interactions.

**METHODS**

**Viruses, VLP expression and purification.** Recombinant baculoviruses containing cDNAs encoding Norwalk virus VP1 and VP2 or Snow Mountain virus VP1 and VP2 have been characterized and described previously (Jiang et al., 1992; Lochridge & Hardy, 2003). Spodoptera frugiperda (SB) cells at a density of 2–5×10^6 cells ml^-1 were infected with either rSMV or rNV baculoviruses at m.o.i. values of 1–5 p.f.u. per cell. VLPs were purified from the medium at 5–7 days post-infection by methods previously described (Hardy et al., 1995; Lochridge & Hardy, 2003). Briefly, rVLPs in the supernatant were concentrated by ultra centrifugation for 2 h at 26,000 r.p.m. in a Beckman SW28 rotor and then centrifuged through a CsCl gradient (0–39 g ml^-1) for 18 h at 35,000 r.p.m. in a Beckman SW55 rotor. The band containing rVLPs was collected and concentrated by centrifugation for 2 h at 26,000 r.p.m. in an SW28 rotor. VLP purity and integrity were evaluated by SDS-PAGE and electron microscopy, and protein was quantified by the BCA assay (Pierce).

**Hybridoma generation.**

**Immunizations.** VLPs diluted in PBS were emulsified in TiterMax Gold adjuvant (Sigma). BALB/c mice (Charles River) were injected intraperitoneally with 500 µg VLPs in 100 µl on day 1 and again 2 weeks later. Mice were bled 10–14 days after the second injection and the serum was tested by dot blot for reactivity to VLPs. The final immunization was administered intravenously 3 days prior to the fusion.

**Fusions.** Immune splenocytes (1×10^9) were collected by centrifugation with exponential phase Sp2/0-Ag14 cells at a ratio of 2:1 spleen : myeloma cells in RPMI 1640. The medium was aspirated and cell pellets were overlaid with RPMI 1640 containing 10 mM HEPES, 10% DMSO and 50% PEG-4000. The pellet was dispersed into small clumps over a 3 min period and cells were collected by centrifugation for 6 min at 650 g. Suspended cells were diluted in HAT medium and plated in 48-well plates with peritoneal feeder cells from non-immunized BALB/c mice. Hybridoma supernatants were tested for antibody production by dot blot 10–14 days later. Positive clones were subcloned in HT medium containing 20% fetal bovine serum and plated in 96-well plates. Positive hybrid cells identified by reactivity to VLPs and by relevant functional assay were expanded and cultured in roller culture bottles for antibody purification.

**Haemagglutination and haemagglutination inhibition.** Red blood cells for haemagglutination assays were prepared as previously described (Hutson et al., 2003). Whole blood (types O, A and B) was collected and suspended in two volumes of sterile Alsever’s solution [2-05 % glucose (w/v), 0-8 % sodium citrate, 0-055 % citric acid, 0-42 % sodium chloride, pH 6-1] and store at 4 °C until use. The red blood cells (RBCs) were washed and packed by diluting 1 ml cells in 14 ml PBS (pH 7-4) and centrifuged for 15 min at 500 g. Immediately prior to the assay, packed RBCs were suspended in 0-85 % saline to a final RBC concentration of 0-5 %. Haemagglutination assays were performed as described by Hutson et al. (2003). Purified VLPs were serially diluted twofold in PBS-H (0-01 M sodium phosphate, 0-15 M sodium chloride, pH 5-5; sterile filtered using 0-2 µm pore-size filter) on ice with a starting concentration of 500 µg ml^-1. Diluted VLPs (50 µl) were added to the wells of 96-well V-bottomed plates and an equal volume of 0-5 % RBCs was added. The plates were then gently agitated, covered and incubated at 4 °C for approximately 2 h or until RBCs in negative controls had settled. Hybridoma supernatants were screened for haemagglutination inhibition activity by combining 50 µl of each supernatant with 50 µl of 50 µg VLPs ml^-1 prior to addition of RBCs. Haemagglutination inhibition assays with partially purified mAbs were performed in the same way with serial dilutions of antibody pre-incubated with VLPs prior to addition of RBCs.

**Phage-displayed oligopeptide library screens.** Approximately 10^13 p.f.u. of phage library J404 (Burritt et al., 1996) in 75 µl TBS
Pelleted phage were pelleted in a microfuge for 15 min at maximum speed and then suspended in 50 μl TBS. mAb–phage complexes bound to the beads were eluted with 1 ml 0-1 M glycine, pH 2-2, and collected in a sterile microfuge tube. This first-round eluate was amplified by infecting 5 ml K91 cells in late log or early stationary phase in soft agar in a 9×13 inch baking dish. Plaques were eluted with 40 ml TBS and concentrated by centrifugation for 20 min at 10,000 r.p.m. in a Sorvall SS34 rotor. The supernatant was transferred to a fresh tube and the centrifugation was repeated to remove residual cells. The first-round phage-containing supernatant was collected and phage were precipitated with 0-15 vols PEG/NaCl solution (16-7 % PEG-8000, 0-5 M NaCl). Precipitated phage were concentrated by centrifugation and suspended in 1 ml TBS and 0-15 vols PEG/NaCl. This mixture was centrifuged for 2 min at maximum speed in a microcentrifuge and the resulting pellet was suspended in approximately 300 μl TBS.

Second-, third- and fourth-round selections were performed in the same way and fourth-round phage titres were determined by plaque assay.

Characterization of mAb-selected phage. Fourth-round phage clones were transferred to 2 ml 2×YT medium supplemented with 75 μg kanamycin ml⁻¹ and incubated for 12 h at 37 °C. A sample of 1-7 ml of the cultures was centrifuged for 5 min at maximum speed in a microcentrifuge. PEG-8000 and 5 M NaCl were added (188 μl each) to the supernatant and the samples were placed on ice for 2 h. The precipitated phage were pelleted in a microfuge for 15 min at maximum speed and then suspended in 50 μl TBS.

Two assays were used to identify phage that reacted with mAbs. Phage that reacted with anti-rNV mAb 54.6 were identified by dot blot. Purified phage clones were spotted onto nitrocellulose and membranes were probed with mAb 54.6, followed by peroxidase-conjugated goat anti-mouse IgG. The blots were developed with ECL Detection Reagent (Amersham Biosciences). Phage that reacted with anti-rSMV mAb 61.21 were identified by ELISA. Purified phage were adhered to the wells of a 96-well Immulon-1B ELISA plate by incubation overnight at 4 °C. The wells were blocked for 45 min at room temperature with 100 μl PBS containing 3 % BSA. mAb 61.21 was added to each well at a concentration of 1-5 μg ml⁻¹ diluted in PBS and incubated for 2 h at room temperature. The wells were washed three times with PBS/0-05 % Tween 20 and peroxidase-conjugated goat anti-mouse IgG was added to each well and incubated for 45 min at room temperature. After washing three times with PBS/Tween 20, the reactions were developed with orthophenylenediamine dihydrochloride substrate and the absorbance read at 490 nm on an ELISA plate reader.

Immunohistochemistry. Differentiated Caco-2 cells (ATCC HTB-37) were prepared by culture to 14 days post-confluence as previously described (White et al., 1996). Cells were collected by low-speed centrifugation and washed several times with PBS. Washed cell pellets were suspended in OCT medium and then snap frozen in liquid nitrogen. The frozen cell blocks were released from the microfuge tube and mounted onto a tissue block with OCT medium. Sections (5 μm) were cut and mounted on SuperFrost Plus Charge slides (Fisher).

Cell sections were fixed with 75 % acetone/25 % ethanol for 5 min and rinsed three times with rinse buffer (Dulbecco’s PBS, 0-2 % goat serum, 0-05 % Tween 20). Cell sections were then blocked with hydrogen peroxide for 10 min, rinsed twice and blocked with avidin and biotin for 15 min each. Sections were incubated with 5 μg rNV ml⁻¹ and then rinsed and incubated with specific mAb or isotype-matched control mAb for 30 min. In blocking assays, particles were incubated with mAbs for 30 min prior to addition to the sections. The sections were rinsed and incubated for 30 min with biotin-conjugated goat F(ab’)₂ anti-mouse Ig (BioSource) diluted 1:250 in 10 % goat serum containing 0-05 % Tween 20. UltraTak strepavidin–horseradish peroxidase substrate (BioSource) diluted 1:500 in rinse buffer was added to the sections and incubated for 20 min. Sections were rinsed once and aminoethylcarbazole chromogen (Dako Cytomation) was added for 1–3 min. Sections were rinsed with distilled water and counterstained with haematoxylin.

RESULTS

Anti-rNV and anti-rSMV mAbs block haemagglutination

Several amino acid residues in the P2 domain of VP1 and domains assembled by the interactions of VP1 dimers in the intact virion have been proposed to be important in epithelial cell and carbohydrate binding (Hardy et al., 1996; Tan et al., 2003, 2004; White et al., 1996). To delineate specific domains on norovirus capsids that may be important in interactions with cellular receptors and to identify epitopes that may elicit neutralizing antibodies in vivo, hybridomas secreting antibodies to rNV and rSMV VLPs were generated and screened for the ability to inhibit haemagglutination and to block binding of VLPs to differentiated Caco-2 cells.

Hybridoma supernatants were first screened by dot blot for reactivity with rNV. Most supernatants showed positive reactivity of varying strengths with purified rNV particles (data not shown). As these studies were in progress, the ability of norovirus VLPs to agglutinate RBCs and bind carbohydrate antigens was reported (Hutson et al., 2003; Marionneau et al., 2002). We adapted the haemagglutination assay to test hybridoma supernatants for blocking antibodies as a rapid screening format. Anti-rNV mAb 54.6 successfully blocked the ability of rNV particles to agglutinate type O RBCs in a dose-dependent manner (Fig. 1) and up to a particle concentration of 100 μg ml⁻¹ (not shown). rNV haemagglutinates types A, AB and O (Hutson et al., 2003). In our assays, a mean of 15 ng rNV particles agglutinated type O RBCs. Agglutination with type B RBCs also occurred, but required a much higher amount of VLPs than the standard assay (data not shown), consistent with results reported by Hutson et al. (2003).

Hybridoma supernatants were tested for reactivity to rSMV particles by dot blot, and positive supernatants were then tested for the ability to block agglutination of type B RBCs by rSMV particles. mAb 61.21 blocked haemagglutination in a dose-dependent manner (Fig. 1) and to a concentration of rSMV of 100 μg ml⁻¹ (not shown). Two studies have reported the carbohydrate-binding specificity of rSMV particles in saliva assays or in assays with synthetic carbohydrate
antigens (Harrington et al., 2002, 2004). In these studies, rSMV particles bound predominantly to type B, but not to types O or A. In our assays, 15 ng rSMV agglutinated type B RBCs. Agglutination of types A and O was observed, but again required much higher concentrations of VLPs.

One of the first characterized mAbs to rNV particles could block specific binding of VLPs to differentiated Caco-2 (D-Caco-2) cells (White et al., 1996). We tested whether the ability of anti-rNV mAb 54.6 to inhibit haemagglutination correlated with an ability to block VLP binding to D-Caco-2 cells as evaluated by immunohistochemistry. In the absence of blocking antibody, rNV bound uniformly to D-Caco-2 cell sections along the cell periphery, consistent with membrane staining (Fig. 2a). Binding was quantified by counting the number of fields with positive stain in at least 50 fields. Incubation of mAb 54.6 with rNV particles prior to addition to cell sections reduced binding by a mean of 94% between multiple experiments (Fig. 2b). These data suggest that mAb 54.6 may have similar capsid recognition properties to the previously reported mAb 8812, although competition assays between these mAbs have not been performed. The binding specificity of rSMV to Caco-2 cells was not as clear as that observed for rNV. Therefore, anti-rSMV mAb 61.21 blocked haemagglutination, but its activity toward rSMV binding to epithelial cells remains to be determined.

Anti-rNV mAb 54.6 and anti-SMV mAb 61.21 recognize conformational epitopes

The nature of the epitopes to which anti-rNV and anti-rSMV antibodies are directed was determined. VLPs were either denatured by boiling in SDS sample buffer or not denatured prior to SDS-PAGE and immunoblot analysis with the respective mAbs. Reactivity to capsid protein by each of the mAbs was detected only in samples that were not denatured, indicating that both antibodies recognize conformational epitopes present in hetero-oligomeric form and possibly representing dimers (Fig. 3).

Sequence and structure analysis of the mAb 54.6 epitope

We screened a phage-displayed random nonapeptide library with mAbs 54.6 and 61.21 to identify epitopes recognized
by these mAbs and, by extension, identify regions on noro-
virus capsids important for cell interactions. Four rounds of
selection were performed to select high-affinity peptides.
Twelve phage clones selected by mAb 54.6 were sequenced
and contained inserts that translated to the peptide
WTRGDHILH. No more than two sequential amino acids
derived from the 54.6-selected sequence could be matched
to the linear VP1 sequence, an observation not surprising
given the conformational nature of the epitope. The peptide
selected by mAb 54.6 contained an RGD sequence, an
integrin-binding motif that plays a role in receptor–ligand
interactions for foot-and-mouth disease virus and adeno-
virus, among others (Mason et al., 1994). Tan et al. (2003)
aligned VP1 amino acid sequences of 11 norovirus strains
and identified an RGD-like motif at aa 291–293 in the P2
domain of VP1 (NV strain numbering) that was moderately
conserved across GI and GII, although there was variation at
the third position and lysine sometimes substituted for
arginine at the first position. The sequence in NV VP1 that
corresponds to the RGD-like motif is RGT (Fig. 4). Mapping
of this putative epitope to the crystal structure of rNV showed its location in the distal P2 domain of the
capsid (Fig. 5). A threonine residue at aa 302 could reflect a
discontinuous TRG sequence corresponding to the mAb
54.6 epitope. The clustering of Thr-302 with the RGD-like
motif supports a potential role of this residue in formation
of a discontinuous conformational epitope recognized by
mAb 54.6.

Sequence and structure analysis of the mAb 61.21 epitope

Three phage clones selected by mAb 61.21 were sequenced
after four rounds of affinity selection and contained inserts
that translated to WLPAPIDKL. This peptide inversely
matched six sequential amino acids in SMV VP1 from D318
to L323 (Fig. 4). These residues were a direct match, but
mAb 61.21 did not bind denatured rSMV VP1 in immuno-
blots, suggesting that, although the amino acids are in linear
sequence, a conformation not present in denatured VP1 is
required for antibody binding. Some of the amino acid
residues that constitute the epitope are part of a motif that is
completely conserved among noroviruses and consists of
PAPXGXPD. Bovine and swine strains show variation only
by the absence of the second proline residue (Fig. 4). The
61.21 epitope was modelled on to the crystal structure of
rNV and displayed close spatial proximity to the putative
54.6 epitope (Fig. 5). Most of the epitope was visible from
the outside and appeared to be located in a surface depres-
sion or cavity of the structure.

Norovirus VP1 cell-binding epitopes

Fig. 3. Anti-rNV mAb 54.6 and anti-rSMV mAb 61.21 recog-
nize conformational epitopes. rNV and rSMV particles were
either boiled (B) or not boiled (NB) in SDS-PAGE sample
buffer and analysed by immunoblot probed with either mAb
54.6 or mAb 61.21. Signals were detected by chemilumines-
cence. Sizes are given in kDa.

Fig. 4. Amino acid sequence alignment of VP1 from norovirus strains representing the
major genogroups in the region correspond-
ing to predicted epitope locations. Aa
238–337 (NV strain numbering) of VP1
were aligned using CLUSTAL W. Conserved
residues are indicated by asterisks, residues
of similar size and hydropathy are indicated
by colons and residues where the size or
hydropathy has been preserved are indicated
by dots. Predicted NV and SMV epitopes
are boxed. Strains: Toronto, Mexico (MXV),
Snow Mountain (SMV), Hawaii (HV), swine
virus isolate 43 (Swine), Lordsdale (LV),
Bristol, Grimsby (GrV), Alphatron, Chiba,
Southampton (SOV), HSS/3/97/DEU (HSS),
Norwalk (NV), Desert Shield (DSV) and Jena
Bovine (Jena).

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Sequence conservation of residues involved in the 61.21 epitope led us to question whether mAb 61.21 recognized VLPs derived from other norovirus strains. Assays with recombinant VLPs representative of both major genogroups including Southampton (GI.2), Desert Shield (GI.3), Toronto (GII.3), Lordsdale (GII.4) and MD-145 (GII) failed to reveal cross-reactivity when assayed by dot blot (data not shown).

**DISCUSSION**

Classical virus neutralization assays are not possible for human noroviruses without a cell-culture system. If it is generally accepted that carbohydrate-binding assays are physiologically relevant, then the ability of VLP-immunized mouse sera to block binding to carbohydrate in saliva-binding assays (Harrington et al., 2002) suggests that antibodies induced by VLPs must at least partially reflect neutralization properties. We are interested in defining sequence and structural requirements that are important in VLP interactions with host cells. Although the blocking activity reported here does not technically constitute neutralization by the classical definition, clearly these mAbs are inhibiting initial interactions with cells and we have defined the first capsid epitopes that play a role in this process.

Anti-rNV mAb 8812 from a previous study blocked binding of rNV particles to D-Caco-2 cells and reacted with a VP1 mutant that contained aa 300–384 of the P2 domain (Hardy et al., 1996; White et al., 1996). Evidence of a role for the P2 domain of VP1 in cell binding has been provided by studies with animal caliciviruses that can be cultivated (Matsuura et al., 2001; Neill et al., 2000; Tohya et al., 1997) and recently for norovirus strains that infect humans by *in vitro* carbohydrate-binding assays or by inhibition of haemagglutination. We have taken advantage of VLP interactions with both epithelial cells and RBCs and have characterized epitopes recognized by mAbs that block binding.

Peptide mimics of the epitopes recognized by mAbs 54.6 and 61.21 were isolated by screening a random nonapeptide phage-displayed library. Reactivity of both mAbs to VP1 was strictly conformation dependent. Unanchored synthetic peptides corresponding to those selected by the antibodies failed to react with mAbs, suggesting that these epitopes are presented in appropriate conformational context on the phage particle when constrained by N- and C-terminal flanking amino acid residues, but not in the free peptides. Despite its conformational dependence, the epitope selected by mAb 61.21 was readily identified because six of the nine amino acid residues in the peptide matched a linear sequence in SMV VP1. Identification of the precise epitope recognized by mAb 54.6 remains speculative, because we could not match peptide residues to those in NV VP1. Selection of such a mimotope by phage display that does not resemble the protein sequence is not uncommon and simply reflects the conformation dependence of antibody recognition. Based on the 54.6 peptide sequence, sequence and structural analyses of VP1, gross mapping of another blocking mAb and putative binding sites reported by others, a potential binding site of mAb 54.6 can be proposed. One potential site contains an RGD-like motif that is moderately conserved among noroviruses. The importance of this motif in carbohydrate recognition was suggested by data showing that mutation of this region in VLPs of two norovirus strains abolished binding to A, B and H antigens in saliva-binding assays (Tan et al., 2003). If this site is indeed the epitope recognized by mAb 54.6, then the mechanism of haemagglutination inhibition and inhibition of binding to epithelial cells may be a direct blocking of the cell-attachment site on the VLP.

The peptide selected by mAb 61.21 matched six continuous residues in VP1 from aa 318 to 323 of the P2 domain. Chakravarty et al. (2005) recently reported sequence and structural analysis of numerous norovirus strains using the evolutionary trace method and identified amino acid residues in VP1 that are absolutely conserved across the genus. Residues of the epitope selected by mAb 61.21 comprised a portion of a motif that is invariant among all noroviruses according to this classification method. The fact that a portion of the epitope recognized by mAb 61.21 is completely conserved among norovirus strains, yet mAb 61.21 reactivity was SMV specific with respect to the other
norovirus VLPs tested, suggests that the epitope also contains amino acid residues that are variable among strains. The mAb recognition site may be constructed by a combination of functionally conserved residues that assemble a structural landscape upon which variable residues coordinate. These variable residues may dictate the narrow antigenic reactivity of mAb 61.21. This hypothesis correlates well with hypotheses put forth regarding how the functional, i.e. cell-recognition structures, of viral capsids are conserved in the presence of immune selective pressure (Stewart & Nemerow, 1997). Since binding of mAb 61.21 to rSMV VLPs did not disrupt the particles (data not shown), structural analyses with F(ab)2 fragments bound to VLPs are possible and will shed additional light on the location and structure of this epitope.

We identified a putative blocking antibody-recognition site on rNV particles and an unambiguous site on rSMV particles. We cannot conclude at this time that these sites are directly responsible for viral attachment, because of the strict conformational dependence that resulted in the inability of free synthetic peptide to compete with VLPs for cell binding. Thus, it is possible that steric hindrance or conformational changes in the capsid introduced by binding of the antibody resulted in a loss of cell binding, rather than mAb binding to the actual site. Mutational analyses of VP1 targeted to proposed residues involved in each epitope were not immediately performed because of interpretive issues associated with loss of function versus loss of structural integrity that would not be recognized at the resolution of standard electron microscopy. Ongoing studies focus on alternative methods of presentation of each of these epitopes that will retain conformation dependence and allow extensive mutational analyses.

We have identified the first amino acid residues of VP1 recognized by a function-blocking mAb. The fact that an antibody that inhibits the ability of rSMV particles to haemagglutinate recognizes an epitope containing amino acids that are completely conserved in every norovirus described so far suggests that this region is structurally important and could be a target for development of cell-binding inhibitors. The domains recognized by these blocking mAbs coincide closely with those recently suggested by others to be important in cell attachment, and our data support predictions of the distally located P2 domain as the primary domain that contains sites important for cell binding.

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