Identification of an antibody-binding epitope on the rotavirus A non-structural protein NSP2 using phage display analysis

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The non-structural protein 2 (NSP2) of rotavirus has important roles in rotavirus replication associated with RNA binding, hydrolysis of NTPs and RNA, and helix destabilizing properties. A cell-culture assay using an NSP2-specific mAb and polyclonal antiserum to block virus replication showed a 73 and 96 % reduction in the amount of virus produced during replication, respectively. Phage display technology was used to identify the antibody-binding region on the NSP2 protein with the motif 244 T-(Y/F)-Ø-Ø-Ø-X-K-Ø-G 252 , where Ø is a hydrophilic residue and X is any amino acid. This region was mapped to the three-dimensional NSP2 crystal structure to visualize the epitope. Analysis revealed identity to a region on NSP2 that mapped to a site exposed on the surface of the protein, which could possibly interfere with a functionally important region of the protein. Antibody binding to this region could disrupt the essential roles of NSP2, such as the formation of viroplasms with NSP5 or the interaction with viral RNA, thereby indicating a possible mechanism for the observed inhibition of virus replication. Genetic analysis of the putative binding region of NSP2 revealed a high level of conservation, suggesting that the region is under strict control.

INTRODUCTION

Rotavirus A (RV-A) is the leading cause of acute gastroenteritis in young children. Annually, the virus causes an estimated 600 000 deaths due to severe dehydration (Parashar et al., 2006). Natural immunity is acquired after early exposure, and confers protection against subsequent severe disease but does not prevent reinfection. The correlates of protection from RV-A infection and disease are not fully defined, and this signifies a limitation in the development of second- and third-generation vaccines (Desselberger & Huppertz, 2011; Franco et al., 2006; Ward, 2008, 2009).

Rotavirus non-structural protein 2, NSP2, is the earliest and most abundant protein present in a cell after rotavirus infection, with both serum and intestinal antibodies detected against this protein (Colomina et al., 1998; Kirkwood et al., 2008; Svensson et al., 1987a). NSP2 is a 35 kDa monomer that self-assembles into homo-octamers in the cytoplasm of infected cells (Jiang et al., 2006). It interacts with another non-structural protein, NSP5, early in the replication cycle to form large cytoplasmic viroplasms, which are the sites of genome replication and viral particle packaging (Eichwald et al., 2004; Fabbretti et al., 1999, 2007). NSP5 exists in several phosphorylated isoforms and is proposed to recruit viral protein to viroplasms (Afrikanova et al., 1998; Contin et al., 2010; Poncet et al., 1997). The interactions of these two proteins are essential to viroplasm formation; co-expression of these proteins in uninfected cells results in viroplasmic-like structures, and blocking of either protein leads to reduced viroplasm formation (Fabbretti et al., 1999; Taraporewala et al., 2002; Vascotto et al., 2004). In addition to forming viroplasms, NSP2 has other important roles in the replication cycle.

Electropositive grooves that encompass a histidine triad (HIT)-like motif on the protein allow the non-specific binding of ssRNA and hydrolysis of the γ-phosphate from NTPs and RNA, possibly acting as a molecular motor (Schuck et al., 2001; Taraporewala et al., 1999, 2006;
NSP2 also has helix destabilizing properties, proposed to remove RNA–RNA duplexes formed in viral mRNA. Such duplexes could prevent translation and replication, and it follows that NSP2, interacting closely with the viral core structural protein VP2, is likely to be involved in the regulation of genome replication through duplex removal (Berois et al., 2003; Taraporewala & Patton, 2001; Taraporewala et al., 2002; Vende et al., 2003).

Studies have shown that NSP2-specific IgA and IgG antibodies are present in >75% of children after natural RV-A infection (Kirkwood et al., 2008; Svensson et al., 1987a, b). Immune responses to NSP2 may contribute to the development of protection against severe clinical disease during reinfection by blocking one or more functions of NSP2 in virus replication. In this study, we have described in vitro cell-culture assays using NSP2-specific monoclonal and polyclonal antisera that are able to block RV-A replication. Further studies were conducted by phage display analysis to localize the immunologically reactive epitope of NSP2, and protein modelling analysis was undertaken to help resolve the position of the NSP2 antibody epitope within the protein structure.

**RESULTS**

**Blocking of RV-A replication with NSP2 antibodies**

To investigate whether antibodies targeting NSP2 can inhibit RV-A replication, we conducted a series of in vitro blocking experiments. Virus titres of RV-A strain SA11 were determined with and without antibody addition. As depicted in Fig. 1, the virus titre of SA11 in MA104 cells was significantly reduced after the addition of anti-NSP2-specific polycrystalline serum, resulting in a 95.6 ± 1.6% reduction in virus titres when compared with the virus titres obtained without antibody. Similarly, addition of NSP2-specific mAb to SA11 resulted in a reduction in virus titre of up to 72.8 ± 6.65%. The addition of rabbit anti-SA11 antiserum completely blocked virus replication. The addition of pre-immune serum did not interfere with virus replication. Virus titres did not differ when the antibodies were added to the cells with the virus, to the cell maintenance medium following infection or to both, suggesting that the antibody was not binding directly to the virus (results not shown).

**Western blotting of cell-culture-grown virus with anti-NSP2 mAb**

To investigate the ability of the anti-NSP2 mAb to bind to native NSP2 protein, Western blot analysis was conducted. NSP2 protein from strains of the two main human RV-A genogroups, Wa and DS-1, with NSP2 genotypes representing N1 and N2, as well as the animal genotype N5 (strain SA11) were analysed. The anti-NSP2 mAb showed reactivity to the NSP2 protein from all virus strains analysed and did not show any reaction in the uninfected MA104 cell culture control (results not shown).

**Phage display**

To identify the antibody-binding epitope on NSP2, a 20mer random peptide library was screened using multiple rounds of panning against the anti-NSP2 mAb immobilized on microtitre plates. The peptides were displayed on the phage minor coat protein, pIII, with five copies of the peptide per phage particle. The phage library had an estimated diversity of $10^9$ peptide clones (Casey et al., 2004; Scott & Smith, 1990). The phage library underwent six rounds of selection panning on the anti-NSP2 mAb, where the eluted phage from one panning round was amplified and used for consecutive panning rounds. The wash stringency was increased with each panning round to select peptides with high antibody-binding affinity. After two rounds of panning, phage pools displayed increased binding to the anti-NSP2 mAb but not to an IgG1 isotype control (mAb 5G8) or to coating buffer. Eluted phage from each round of panning underwent ELISA analysis to determine the enrichment of the library for binders to the anti-NSP2 mAb (Fig. 2).

The library showed an increase in the proportion of phage that bound to the anti-NSP2 mAb from panning rounds 4–6. Three distinct peptide sequences were identified in the third round of panning (clones 3.1, 3.2 and 3.7; see Supplementary Table S1, available in JGV Online); however, no single consensus sequence was apparent for the round 3 clones. Sequence analysis of a further 22 clones from panning rounds 4–6 all resulted in sequences identical to clone 3.7 (see below).
Relative binding affinity of clone 3.7

The relative binding affinity of three clones isolated from panning round 3, each with a unique peptide sequence (clone 3.1: AKAFSYWTPFDGKSLYSST; clone 3.2: IVTAR-ARHDWNWVQRSSRSL and clone 3.7: TYNHDYKTGM-VIYSPFMTYP) were compared at the same concentration by ELISA. Two of the clones, 3.1 and 3.7, bound to the anti-NSP2 mAb, with clone 3.7 binding at a significantly higher level than 3.1. Both clones showed a low binding affinity to an antibody isotype control (mAb 5G8). Clone 3.2 bound at very low levels to both anti-NSP2 mAb and the isotype control (Fig. 3).

The peptide 3.7 motif and NSP2 sequences

A BLAST search using the Swiss-Prot protein sequence database with rotavirus search parameters (taxid: 10912) showed a region of identity between the amino acid sequence of phage clone 3.7 and the human RV-A strain KU (GenBank accession no. AB022770) at aa 244–255. Amino acid residues T244, Y245, K250, G252, V254 and I255 were identified as being conserved between the KU NSP2 protein sequence and the peptide sequence (Fig. 4a). In a ClustalW2 alignment with RV-A strains SA11, Wa, KU, UK, DS-1 and RV5, the phage clone 3.7 peptide sequence exhibited identity to two short domains, aa 244–252 and 263–273. These strains were obtained from GenBank and had NSP2 genotypes of N5 (SA11), N1 (Wa, KU and UK) and N2 (DS-1 and RV5) (Matthijssens et al., 2008). Three of the nine residues in the first domain (T244, K250 and G252) and a single residue in the second domain (I265) were identical between the RV-A strains and the peptide sequence. In the first region of the alignment between the NSP2 protein sequences and the peptide clone 3.7, there were four positions (aa 246–248 and 251) that contained only hydrophilic amino acids (Fig. 4b). It could be that the antibody-binding epitope consists of three key residues (T, K and G) in a hydrophilic protrusion forming the motif 244T-(Y/F)-Ø-Ø-Ø-X-K-Ø-G252, where Ø are hydrophilic residues and amino acids at position X249 did not display conserved/similar properties.

A high level of sequence variation between conserved sites was observed in the first domain, whereas the second domain showed conservation in the strains analysed. The 244T-(Y/F)-Ø-Ø-Ø-X-K-Ø-G252 motif was found to be conserved in an alignment of 228 human NSP2 sequences from GenBank and Australian RV-A isolates (results not shown). Overall rates of genetic distances in this dataset were measured at a minimum of 71.12 % nucleotide and 87.14 % amino acid similarity.

Protein modelling

Protein modelling studies of NSP2 were undertaken to localize the antibody-binding region identified by phage display. Using the protein modelling program PyMol (DeLano, 2002), the anti-NSP2 mAb epitope was mapped to the SA11 NSP2 protein structure obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB, http://www.rcsb.org/pdb/home/home.do; ID 119V) (Jayaram et al., 2002). The NSP2 antibody-binding epitope region spanning aa 244–252 was
located in the C-terminal domain of the NSP2 protein, in a region protruding from both the monomer and the molecule in octamer formation (Fig. 5). In contrast, the second region identified by phage display spanning aa 263–273 was located within the backbone of the NSP2 protein structure. The internal location of region two in the NSP2 protein, combined with the low level of identity between the NSP2 protein and peptide sequences, suggested that this region is unlikely to represent an immune epitope and is an artefact of the 20mer length of the peptide sequence.

The active sites for the NTPase functions of NSP2 and interaction with NSP5 were mapped onto the protein model and compared with the location of the antibody-binding epitope. The NTPase function of the protein is carried out by the HIT motif that lies in the cleft between the two domains of NSP2 (Vasquez-Del Carpio et al., 2004, 2006) and falls within close proximity of the antibody-binding epitope identified. Mapping the regions of interaction between the NSP2 and NSP5 proteins and the antibody-binding epitope showed that these two regions fall near to each other and share residues (aa 244–251).

**DISCUSSION**

NSP2 is a non-structural viral protein that is produced early in the rotavirus replication cycle. A study using a heat-sensitive mutant has shown that this protein is essential for virus replication (Chen et al., 1990; Taraporewala et al., 2002). Further studies have indicated that NSP2 has several important roles, such as helix destabilization, ssRNA binding, NTPase/RTPase functions and the ability to form inclusion bodies (Jayaram et al., 2002; Jiang et al., 2006; Kumar et al., 2007; Schuck et al., 2001; Taraporewala & Patton, 2001; Taraporewala et al., 1999, 2002, 2006; Vasquez-Del Carpio et al., 2004, 2006). In this study, we have presented data indicating that NSP2-specific antibodies (monoclonal and polyclonal) can block RV-A replication in an in vitro cell-culture assay. Using phage display analysis, we were able to localize the antibody-binding epitope for the anti-NSP2 mAb to aa 244–252, which is located on a finger-like protrusion at the C-terminal end of the NSP2 protein.

The blocking of RV-A replication by anti-NSP2-specific monoclonal and polyclonal antisera provides preliminary evidence that antibodies targeting this protein could play a role in the protection seen during RV-A infection. Heterotypic antibodies targeting NSP2 have been detected in children following RV-A infection (Kirkwood et al., 2008; Svensson et al., 1987a) and the ability of the anti-NSP2 mAb used in this study to bind to NSP2 from a variety of genetically distinct RV-A strains supports this concept. The correlates of protection from RV-A infection and disease are not fully defined and the debate remains open on possible determinants of this protection (Desselberger & Huppertz, 2011). The outer capsid proteins, VP4 and VP7, are the primary focus for vaccine development, and neutralizing antibodies targeting these proteins have been identified; however, these responses do not prevent RV-A reinfection, suggesting that other structural and non-structural proteins may be involved (Ray & Kelkar, 2004; Ward et al., 1990, 1993). Previous studies have shown that serum and intestinal antibodies targeting NSP2 rise to high titres following infection and reinfection (Colomina et al., 1998; Kirkwood et al., 2008; Svensson et al., 1987a). Together with the in vitro cell-culture assay described in this study, where anti-NSP2 antibodies were able to interfere

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**Fig. 4.** (a) BLAST analysis of the phage clone 3.7 in the Swiss-Prot database reveals similarity to the NSP2 protein sequence of RV-A strain KU in aa 244–255. (b) Sequence alignment of RV-A NSP2 proteins in the region of the anti-NSP2 mAb epitope. The aligned sequences included representatives from the two major RV-A NSP2 genotypes N1 (Wa, KU) and N2 (DS-1, RV5 and UK), as well as the simian strain SA11 (N5). Alignment was constructed in ClustalW2 and defined the epitope motif 244- T-X-Ø-Ø-Ø-X-K-Ø-G 252 , where X 245 was tyrosine or phenylalanine (Y/F) and residues at X 249 did not display conserved properties. Ø represents hydrophilic residues. Dark grey shading indicates identical amino acids; light grey shading indicates conserved hydrophilic amino acids.
with virus replication, it is possible that NSP2 antibodies may play a role in protection, perhaps by a mechanism whereby the antibodies are transported across cell membranes into cells where they can bind to the NSP2 proteins and inhibit replication. Transport across cell membranes of immunoglobulins that interfere with virus replication through polymeric immunoglobulin receptor (pIgR) transcytosis has been described in several virus systems, including Sendai virus (Fujioka et al., 1998; Mazanec et al., 1992) and human immunodeficiency virus (Huang et al., 2005; Wright et al., 2006), as well as against the VP6 protein of rotavirus (Feng et al., 2002; Kaetzel, 2005; Ruggeri et al., 1998; Schwartz-Cornil et al., 2002). Another possible mechanism of inhibition of virus replication is binding of secretory IgA to viral protein in vivo followed by transportation from the basolateral epithelial layer to the apical surface of the cell through the pIgR as an antibody–antigen complex, as has been seen to occur with influenza virus (Kaetzel, 2005; Mazanec et al., 1992; Mostov, 1994). This mechanism could be particularly important during RV-A reinfections, where intracellular neutralization of NSP2 by IgA could inhibit virus replication and reduce the cytolysis of infected cells, thus preventing the sloughing of enterocytes and villi and reducing the onset of diarrhoea and other symptoms as observed with reinfections. It would be of interest to investigate the protective response targeting NSP2 in a mouse model, as rotavirus infection of MA104 cells has been shown to increase cell permeability (Cuadras et al., 1997; Denisova et al., 1999), thus raising the possibility that the anti-NSP2 antibodies entered the infected cells through a compromised cell membrane.

To understand the range of immunological epitopes present on the NSP2 protein, we conducted a series of phage display experiments. After six rounds of panning, a phage clone displaying a peptide with affinity for the anti-NSP2 mAb, peptide 3.7, was selected. BLAST and ClustalW2 analysis of the phage clone 3.7 peptide and NSP2 protein sequences located the motif $^{244}$T-(Y/F)-Ø-Ø-Ø-X-K-Ø-G$^{252}$ in the C-terminal domain of the protein, where Ø is a hydrophilic residue and amino acids at position X did not display conserved/similar properties. This motif was found to be conserved from analysing an alignment of Australian and GenBank human NSP2 sequences, which also suggests that antibodies against NSP2 may provide heterotypic protection.

Investigation of the epitope regions represented by phage clone 3.7 through protein modelling using an established three-dimensional structure (Jayaram et al., 2002) showed that the $^{244}$T-(Y/F)-Ø-Ø-Ø-X-K-Ø-G$^{252}$ motif mapped to a protrusion on the outer edge of the monomer and octamer. This region of the NSP2 protein has been identified as a functional region. The HIT motif, comprised of H$^{221}$ and H$^{225}$ and surrounding residues, catalyses hydrolysis of the terminal phosphate group from nucleotides and RNA, in a function proposed to provide energy for packaging of viral particles (Kumar et al., 2007; Vasquez-Del Carpio et al., 2004, 2006). The HIT motif lies deep within the cleft that separates the N- and C-terminal domains of the NSP2 protein and falls within close proximity of the antibody-binding epitope suggested to occur on the protruding region on NSP2. It is possible that antibodies binding to this region prevent the entry of nucleotides into the cleft, thus preventing the provision of energy for virus packaging, thus inhibiting replication.

During the course of the rotavirus replication cycle, NSP2 interacts with NSP5 forming viroplasms. The viroplasms
form the sites of genome replication and viral particle packaging (Eichwald et al., 2004; Fabbretti et al., 1999; Mohan et al., 2003). NSP5 is thought to interact with the C-terminal domain of the NSP2 monomer (Eichwald et al., 2004; Mohan et al., 2003; Sen et al., 2006), in particular the C-terminal α-helix and the loops between aa 291 and 302, aa 64 and 68 and aa 179 and 183, as well as the helix between residues 232 and 251 (Jiang et al., 2006). The proposed antibody-binding epitope included in aa 244–252, as identified through phage display, aligns exactly with a region on the NSP2 protein that is involved in the interaction with NSP5. Antibody binding to this region of the NSP2 is likely to prevent the interaction with NSP5 and block viroplasm formation, thus inhibiting virus replication by removing the site of genome replication and viral particle packaging.

In conclusion, antibodies targeting the RV-A non-structural protein NSP2 have the ability to interfere with virus replication. Phage display analysis of an NSP2-specific mAb identified a possible epitope motif, 544-T-(Y/F)-Ø-Ø-Ø-X-K-G-252, on a surface protrusion of the protein. Antibody interaction at this region could block several rotavirus functions including viroplasm formation and packaging. These studies provide further insight into the diverse role of NSP2, and provide further evidence of the importance of non-structural proteins.

**METHODS**

**Cells and viruses.** Monolayers of the MA104 cell line were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS. The G3P[I] simian RV-A strain SA11 was cultured in MA104 cells with 10 μg trypsin ml⁻¹ [from porcine pancreas, 14 800 N-benzoyl-l-arginine ethyl ester (BAEE) units mg⁻¹; Sigma-Aldrich] according to previously described methods (Gorrell & Bishop, 1997).

**Antibodies.** Rabbit polyclonal antiserum was prepared by subcutaneous injection of either gradient-purified SA11 or an *Escherichia coli*-produced recombinant NSP2–His fusion protein. The antigens were given as three doses, each 1 month apart, and the polyclonal antiserum was obtained by a terminal bleed 1 month after final immunization. The anti-NSP2 IgG, mAb used for this assay was prepared from the NSP2 recombinant His fusion protein using a miniPERM system through a commercial agreement with the Walter and Eliza Hall Institute (WEHI, Melbourne, Australia).

**Replication blocking assay.** MA104 cells were grown to confluency in 24-well plates and infected with SA11 at a m.o.i. of 0.5 focus-forming units (ff.u.) cell⁻¹. SA11 had been activated with 10 μg porcine trypsin ml⁻¹ for 30 min at 37 °C. Cell monolayers were infected with SA11, SA11 plus anti-SA11 polyclonal antiserum, SA11 plus anti-NSP2 polyclonal antiserum, SA11 plus anti-NSP2 mAb, or SA11 plus pre-immune serum, or were left uninfected for antibody controls. Optimum antibody concentrations were determined to be 2 μg ml⁻¹ after performing a dilution series of the antibodies in replication blocking assays. Trays were incubated for 45 min at 37 °C to allow viral absorption, after which the inoculum was removed and 1 ml maintenance medium (DMEM containing 1 μg porcine trypsin ml⁻¹) was added to each well. Virus replication was allowed to occur for 16 h at 37 °C prior to harvest by three freeze/thaw cycles.

**Titation of virus.** Virus titres were determined by performing serial dilutions of harvested virus in DMEM containing 1 μg trypsin ml⁻¹ in a 96-well plate. The virus was initially diluted 1:5 with DMEM before titration in duplicate in 96-well plates. The plates were incubated for 18 h at 37 °C. After incubation, the supernatant in each well was aspirated and the cells fixed with 75% (v/v) acetone. After removal of the acetone, anti-SA11 polyclonal antiserum, diluted 1:400 with PBS, was added to each well and incubated for 30 min at 37 °C. Cells were washed three times with PBS, and anti-rabbit IgG–FITC conjugate was added to each well and allowed to bind to the anti-SA11 polyclonal antiserum for 30 min at 37 °C. Cells were washed three times with PBS and allowed to air dry. Fluorescent cells were counted using a fluorescence microscope (Leica Microsystems) and virus titres were expressed as f.u. ml⁻¹.

**Western blotting of cell-culture-grown virus.** For analysis of NSP2, virus was cultured for 10 h or until 40% CPE was reached, the medium removed and the cells washed with PBS. The cells were scraped into 750 μl PBS, and 25 μl lysis buffer [100 mM Tris/HCl (pH 8.0), 240 mM NaCl, 0.05% Triton-X] was added. The resuspended cells were vortexed and incubated on ice for 30 min. Large cellular debris was removed by centrifugation at 20 800 g for 6 min. The supernatant was retained and stored at −20 °C. Virus samples, recombinant NSP2 protein (0.1 μg) and uninfected MA104 cell controls were mixed with 5 μl Laemmli loading buffer and boiled for 10 min. Proteins were separated by electrophoresis for 40 min at 200 V and 4 °C. The proteins were transferred to a PVDF membrane, washed twice in TBS and blocked overnight in TBS containing 3% (w/v) skimmed milk powder at 4 °C. The membrane was reacted with the anti-NSP2 mAb diluted 1:10 000 in TBS for 2 h at room temperature. The membrane was subsequently washed in TBS/0.05% Tween (TBS-T) and reacted with HRP-conjugated goat anti-mouse IgG antibody diluted 1:5000 in TBS-T for 2 h at room temperature. Protein-bound antibody was detected using an ECL Western blotting System (GE Healthcare), as specified by the manufacturer, and the signal was visualized by exposure to autoradiography film (GE Healthcare).

**Phage library preparation.** A 20mer random peptide library displayed on the filamentous bacteriophage M13 was used in all phage display experiments. The K91 strain of *E. coli* was used for all phage amplification and for the isolation of individual phage clones. The anti-NSP2 mAb was used for panning experiments. The isotype control antibody was 5G8, a mAb against the malarial apical membrane antigen-1 (AMA1) protein (Coley et al., 2001).

**Phage display.** A 96-well ELISA plate was coated with 1.0 μg mAb ml⁻¹ (anti-NSP2 mAb or isotype control), diluted in coating buffer (0.1 M NaHCO₃) and incubated overnight at 4 °C. The coating buffer/antibody solution was removed with two washes of PBS and the wells were blocked for 2 h with 5% Blotto [5% (w/v) skimmed milk powder in PBS] at room temperature. Approximately 10° of the 20mer phage library, diluted in 1% Blotto, were added to wells that had been washed in PBS and were incubated for 2 h at room temperature. In subsequent panning rounds, the plates were washed with PBS-T (0.05% Tween 20 in PBS) to increase the washing stringency in removing non-binding phage. Antibody-bound phage was eluted using 0.1 M glycine solution (pH 2.2). The phage were pooled and neutralized in a 1.5 M Tris/HCl (pH 9.0), with an aliquot of the eluted library retained for titration. *E. coli* K91 cells were grown to exponential phase (optical density of 0.6 at 600 nm) in 10 ml 2 × YT medium (16% tryptone, 10% yeast extract, 5% NaCl). The K91 culture was held stationary for 10 min to allow F-pilus formation prior to phage addition, and the eluted phage library was then added and incubated for 30 min to allow infection of the K91 cells. Infected K91 cells were added to 200 ml “Super Broth” [30% tryptone, 20% yeast extract, 10% 3-(N-morpholino)propanesulfonic acid]
containing 40 μg tetracycline ml⁻¹ and incubated for 16 h at 37 °C, with shaking at 160 r.p.m. The bacterial cells were pelleted by centrifugation (15 min, 10,000 g) and stored under glycerol at −80 °C. The supernatant was retained and phage were isolated by polyethylene glycol precipitation according to established methods (Adda et al., 1999).

Phage titre determination. Phage were titrated as described previously (Harris et al., 2005). Briefly, tenfold serial dilutions of the phage were performed in 90 μl 2 × YT medium. A 90 μl aliquot of exponential-phase E. coli K91 cells was added to each dilution and incubated at room temperature for 30 min to allow cell infection to occur. A 20 μl aliquot of each phage dilution was spotted onto Luria–Bertani (LB) agar plates containing 40 μg tetracycline ml⁻¹ and incubated overnight at 37 °C. The resulting colonies were counted and expressed as c.f.u. ml⁻¹.

ELISA. Phage ELISA was performed as described previously (Casey et al., 2004). Briefly, a 96-well plate was coated with anti-NSP2 mAb, coating buffer or mAb 5G8, an IgG₁ isotype control. The antibodies were diluted to 5 μg ml⁻¹ in 0.5 M carbonate/bicarbonate buffer and incubated overnight at 4 °C. The plates were washed twice with PBS and blocked with 5 % Blotto for 2 h at room temperature. The Blotto was removed with two washes of PBS before the addition of 10⁻⁶ phage diluted in 1 % Blotto. To check for non-specific binding, phage were also added to wells containing coating buffer only or an isotype control. The plates were incubated for 1 h at room temperature before washing with PBS-T. One hundred microliters of HRP-conjugated mouse anti-M13 diluted 1 : 5000 in PBS was added to each well and further incubated for 1 h at room temperature. The plates were washed with PBS-T and 100 μl o-phenylenediamine was added to the wells. The reaction was halted with the addition of 100 μl M HCl. The absorbance of each well was measured at 490 nm.

Nucleotide sequencing

Peptide inserts. Glycerol stocks of E. coli K91 phage clones from panning rounds 3–6 were streaked out on LB/tetracycline plates (4 %, w/v), grown overnight and subcultured onto LB/tetracycline plates. DNA was released from individual isolates by boiling and the cell debris removed by centrifugation. Random inserts were amplified by PCR, and the cDNA products were used as template in sequence analysis using Big Dye Terminator version 3.1 (Applied Biosystems). The gIII forward primer 5'-GGTCACATAAGCGCTTTCTA-3' and reverse primer 5'-GGCTTTTAAAGCGTC-3' were used to determine the peptide motif displayed on the gIII protein. BLAST searches were performed through the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

NSP2 gene. Rotavirus RNA was extracted from 20 % (w/v) faecal extracts using hydroxyapatite, and the full-length gene encoding NSP2 was amplified by RT-PCR using the RV-A gene 8-specific primers Beg 8 (5'GGGTCTTGAACGTCGCT-3'; nt 1–15) and End 8 (5'GGCTCATAAGGGCTTCTCA-3'; nt 1040–1059). The cDNA products were purified using a QIAquick gel extraction kit (Qiagen) according to the manufacturer’s instructions and used as template for sequence analysis with Big Dye Terminator version 3.1 (Applied Biosystems). The NSP2 gene-specific primers Beg 8 and End 8 were used in sequencing reactions. Sequences were analysed and assembled using Sequencher version 4.6 (Gene Codes). NSP2 sequences were compared with additional sequences obtained from GenBank, and alignments and genetic distances were determined using the ClustalW2 program through the European Bioinformatics Institute (http://www.ebi.ac.uk/) and MEGA5 software (Tamura et al., 2011).

Protein modelling. The protein crystal structure of NSP2 was obtained from the RCSB PDB (ID 1L9V). Areas of interest on the protein were highlighted using the PyMOL Molecular Graphics System (DeLano, 2002).

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