QCM-D studies of human norovirus VLPs binding to glycosphingolipids in supported lipid bilayers reveal strain-specific characteristics

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Susceptibility to norovirus infection has been linked to secretor status. Norovirus virus-like particles (VLPs; 0–20 µg/mL) from the Norwalk (GI.1) and Dijon (GII.4) strains were assayed for binding to H type 1 and Lewis a pentaglycosylceramides, incorporated in laterally fluid supported lipid bilayers. Binding kinetics was monitored in real time in 40 µL stationary reaction chambers, using quartz crystal microbalance with dissipation (QCM-D) monitoring. Both strains displayed binding only to H type 1 and not to Lewis a glycosphingolipids, typical for epithelial cells of susceptible and resistant individuals, respectively. This binding specificity was confirmed by VLPs binding to the two glycosphingolipids chromatographed on TLC-plates. Experiments using bilayers with mixtures of H type 1 and Lewis a, with the total glycosphingolipid concentration constant at 10 wt%, showed that binding was only dependent on H type 1 concentrations and identical to experiments without additional Lewis a. Both strains showed a threshold concentration of H type 1 below which no binding was observable. The threshold was one order of magnitude higher for the Dijon strain (2 wt% versus 0.25 wt%) demonstrating that the interaction with a significantly larger number of glycosphingolipids was needed for the binding of the Dijon strain. The difference in threshold glycosphingolipid concentrations for the two strains suggests a lower affinity for the glycosphingolipid for the Dijon compared to the Norwalk strain. We propose that VLPs initially bind only a few glycosphingolipids but the binding is subsequently strengthened by lateral diffusion of additional glycosphingolipids moving into the interaction area.

Keywords: glycosphingolipid/H type 1/norovirus/QCM-D/
supported lipid bilayer

Introduction

Norovirus has been estimated to cause up to 200,000 deaths of children yearly in developing countries and about half of all outbreaks of gastroenteritis in developed countries (Atmar and Estes 2006; Patel et al. 2008). In lack of effective cell culture methods for virus propagation (Duizer et al. 2004; Guix et al. 2007; Straub et al. 2007), most in vitro studies of the virus have been done using virus-like particles (VLPs). Norovirus VLPs form spontaneously when the capsid protein is recombinantly expressed in insect cells and have similar morphological and antigenic properties compared to native virus (Jiang et al. 1992; Green et al. 1993). Most norovirus VLPs bind to α1,2-fucosylated glycans (Harrington et al. 2002; Marionneau et al. 2002, 2005; Hutson et al. 2003; Huang et al. 2005), a binding specificity that has been confirmed with native viruses (Harrington et al. 2004; Thorven et al. 2005). About 20% of the Caucasian population lack α1,2-fucosylated glycans in mucosal tissues and secretions due to a nonsense mutation in the fucosyltransferase 2 (FUT2) gene (Kelly et al. 1995). Such individuals are denoted nonsecretors and are consequently resistant to most norovirus infections (Lindesmith et al. 2003; Hutson et al. 2005; Thorven et al. 2005; Kindberg et al. 2007; Tan, Jin, et al. 2008). However, different strains show different binding patterns to the ABH and Lewis antigens and some strains even show a FUT2-independent binding pattern (Huang et al. 2005; Shirato et al. 2008). Furthermore, norovirus infections of nonsecretors have been reported (Lindesmith et al. 2005; Rockx et al. 2005), and some nonsecretors have been shown to have significant titers of antinorovirus antibodies (Larsson et al. 2006) indicating that resistance is not absolute but only relative. Additional binding specificities has been reported for human norovirus to heparan sulfate (Tamura et al. 2004) and more recently to sialyl Lewis x and some of its structural analogs (Rydell et al. 2009).

Most norovirus binding studies have made use of saliva of single individuals and of different neoglycoconjugates for mapping the binding specificities of various VLPs. We have, however, shown that VLPs from the Norwalk strain also bind to naturally occurring glycosphingolipids (Nilsson et al. 2009). In the latter study, we used the chromatogram binding assay (CBA) to demonstrate that Norwalk VLPs recognize a large number of FUT2-dependent glycosphingolipids. However, it did not bind to any blood group B-terminated structures, in analogy with the weak recognition of saliva from secretor positive blood group B individuals (Marionneau et al. 2005). Many viruses exploit glycosphingolipids (Karlsson 2001), which may function as true receptors and not only as attachment factors (Tsai et al. 2003; Low et al. 2006). Very recently, it has been suggested that murine norovirus uses siaiylated glycosphingolipids, i.e. gangliosides, as receptors (Taube et al. 2009).

The CBA is an ideal tool when screening for lipid bound carbohydrate ligands in complex biological mixtures or when deciphering the detailed molecular binding specificities of carbohydrate recognizing proteins (Hansson et al. 1984; Karlsson and Stromberg 1987; Johansson et al. 2005). However, the rigid immobilization of the glycosphingolipids on thin layer plates

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or in enzyme-linked immunosorbent assay (ELISA) microtiter wells differs a lot from the fluid environment in the cell membrane. Fluidity is expected to play a key role for receptors of the plasma membrane since after the initial attachment, the binding may subsequently be strengthened by lateral diffusion of additional receptors moving into the area of protein–glycosphingolipid interactions. Furthermore, there is increasing evidence that protein–carbohydrate interactions can be very context dependent, especially when glycosphingolipids are involved (Evans and Roger MacKenzie 1999), and discrepancies regarding glycosphingolipid binding specificities for different assays are indeed found in the literature (Stromberg et al. 1991; Cooling et al. 1995; Lingwood 1996; Kaufmann et al. 2005). An often-used model of cell membranes with maintained fluidity is the supported lipid bilayer (Sackmann 1996), which on certain substrates can be formed spontaneously from lipid vesicle adsorption. One very efficient method to study supported lipid bilayer formation, including subsequent biomolecular binding events, is quartz crystal microbalance with dissipation (QCM-D) monitoring since the obtained response provides unique signatures for successful bilayer formation (Keller and Kasemo 1998; Larsson et al. 2003). Besides measurements of bound mass, which is provided from changes in the resonance frequency, f, of the piezoelectric sensor resonator, the QCM-D technique also provides structural information of biomolecular films via changes in the damping, D, of the crystal (Hook et al. 1998). In this way, adsorbed lipid vesicles, which induce high damping, can be easily distinguished from planar supported membranes, which induce essentially no detectable damping (Keller and Kasemo 1998). The method thus provides real-time label-free monitoring of both successful bilayer formation and kinetics of subsequent binding reactions. Also, via changes in the damping, information on the rigidity of bound entities is provided.

In this study, we have used the QCM-D technique to monitor the binding kinetics of norovirus VLPs to supported lipid bilayers containing H type 1 and Lewis a pentaglycosylceramides. In addition to VLPs from the Norwalk (G I.1) strain, VLPs from the Dijon strain (Nicollier-Jamot et al. 2003) were studied. The Dijon strain belongs to the clinically most common genocluster, GI.4 (Kroneman et al. 2008) and shows a broad secretor gene-dependent binding pattern in saliva and neoglycoprotein assays (Rydell et al. 2009), which is typical for GI.4 strains (Huang et al. 2005; Shirato et al. 2008). The binding of the two VLPs to supported lipid bilayers containing up to 10 wt% of either H type 1 or Lewis a glycosphingolipids were compared, revealing binding specificities in qualitative agreement with the specificities of VLP binding to glycosphingolipids separated on thin layer plates. Differences in the threshold glycosphingolipid concentrations of H type 1, below which no binding was observed for the two VLPs, were evaluated based on binding kinetics and structural properties revealed from combined f and D QCM-D measurements.

Results

Chromatogram binding assay
H type 1 and Lewis a pentaglycosylceramides were chromatographed on TLC-plates and incubated with Norwalk and Dijon VLPs, separately (Figure 1). Bound VLPs were detected using antibodies and alkaline phosphatase (ALP) staining. Anisaldehyde staining revealed only one distinct band in each glycosphingolipid fraction, in agreement with 1H-NMR experiments showing the H type 1 and Lewis a fractions to be >98% pure (Nilssons et al. 2009). Anti-H type 1 and Lewis a antibodies validated that H type 1 and Lewis a epitopes were present only in their respective glycosphingolipid fractions with no cross-reactivity to each other or to any minor components (data not shown). VLP binding assay showed that Norwalk strain only recognized the H type 1 and not the Lewis a glycosphingolipid, in complete agreement with previous studies using radiolabeled Norwalk VLPs (Nilsson et al. 2009). The Dijon VLP showed an identical binding specificity recognizing only the H type 1 but not the Lewis a glycosphingolipid. Binding of VLP to any glycosphingolipids other than H type 1 could not be observed neither in the H type 1 nor in the Lewis a fraction for any of the VLPs. Control plates were run to exclude the possibility of the anti-VLP antibodies cross-reacting with any of the glycosphingolipids but no binding was detected (data not shown).

QCM-D

Bilayer Formation. Glycosphingolipid-containing supported lipid bilayers were formed by exposing SiO2-coated QCM crystals to phospholipid vesicles containing 10 wt% of either H type 1 or Lewis a pentaglycosylceramides. The vesicles were first diluted in a buffer promoting bilayer formation (buffer A), containing CaCl2 (10 mM) and a higher concentration of NaCl (200 mM) than the ordinary Tris-buffer (100 mM NaCl). The formation of the supported bilayer was monitored and representative plots of changes in resonance frequency, f, and energy dissipation, D, versus time are shown in Figure 2. After a steady baseline was achieved in Tris-buffer, buffer A was introduced into the sample chamber. Because of differences in density, ion strength, and viscosity between the buffers, this change caused a decrease in f and increase in D, respectively. These shifts were reversed when the buffer was changed back to the ordinary Tris-buffer, after completed bilayer formation. To initiate the supported lipid bilayer formation process, 500 µL of a lipid vesicle suspension (100 µg/mL total lipid concentration) was pumped through the sample chamber. The lipid vesicle adsorption causes an initial decrease and increase in f and D, respectively, which
saturates at around $-30$ Hz and $0.5 \times 10^{-6}$ after 6 min. Note that both $f$ and $D$ display peaks after around 2 min, which is attributed to the critical coverage of adsorbed nonruptured lipid vesicles at which supported lipid bilayer formation is initiated. The monitored signals are in agreement with a typical bilayer formation process (Keller and Kasemo 1998).

**Binding of Norovirus VLPs.** Norovirus VLPs were introduced into the sample chamber after steady signals from bilayers containing 10 wt% of either H type 1 or Lewis a pentaglycosylceramide were achieved. Because of limited amounts of VLPs, the binding kinetics was monitored in the batch mode with VLP concentrations adjusted to 10 $\mu$g/mL and 20 $\mu$g/mL for Norwalk and Dijon, respectively. Since the concentration of active virus or VLP is hard to determine (Chan et al. 2007; Guix et al. 2007; Straub et al. 2007), the VLP concentration for each strain refers to the total concentration of proteins. However, in all experiments the same amount of VLP was injected into the chamber, allowing for certain quantitative comparisons between measurements (see below). Both the Norwalk and the Dijon VLP bound to bilayers containing 10 wt% of H type 1, whereas no binding was detected for any of the strains to bilayers containing 10 wt% Lewis a (Figure 3). For the Norwalk strain, the final change in $f$ was around $-5.5$ Hz after 6 min, and half of the final response was achieved in less than 1 min. For the Dijon strain, the final change in $f$ was $-19$ Hz after 75 min, and half of the final response was obtained after 3 min. These differences in maximal shifts are most likely primarily attributed to the difference in VLP concentrations. For both strains, more than 65% of the bound VLPs were not released by flowing buffer solution through the chamber (data not shown).

A plot of the changes in $D$ versus $f$, which eliminates time and thus VLP concentration as explicit parameters, is shown for each VLP in Figure 4. Both VLPs showed a linear relationship between $\Delta f$ and $\Delta D (R^2 = 0.99)$. The change in dissipation per change in frequency was for the Norwalk strain $-0.18 \times 10^{-6}$/Hz whereas for the Dijon strain it was $-0.30 \times 10^{-6}$/Hz. The lower $\Delta f/\Delta D$ ratio of the Norwalk VLPs suggests that this strain forms a more rigid structure together with the lipid membrane than the Dijon VLPs. This observation is consistent with, although not on its own a proof of, a tighter adhesion of the Norwalk than the Dijon strain.

To further investigate the binding properties, the VLPs were assayed against bilayers with different densities of H type 1. To compensate for possible domain formations, Lewis a glycosphingolipid was added to achieve a constant total concentration of 10 wt% of glycosphingolipids. Experiments with the Norwalk VLP using bilayers with mixtures of H type 1 and Lewis a, with the total glycosphingolipid concentration constant at 10 wt%, showed however that binding was only dependent on H type 1 concentrations and identical to experiments without additional Lewis a. Bilayers with 0–10 wt% of H type 1 were formed by mixing of vesicles containing 10 wt% of H type 1 and 10 wt% of Lewis a, respectively, in different ratios. An IgG1 antibody directed against H type 1 was included in the analysis to confirm the availability of the H type 1 epitope in the membrane. The
antibody was monitored in the flow mode, whereas the VLPs were monitored in a stationary sample chamber, in analogy with previous VLP experiments. The relations between the wt% of H type 1 in the bilayer and the final change in frequency of the VLPs and of the antibody are presented in Figure 5. The binding of both the Norwalk and the Dijon VLPs was critically dependent on the concentrations of H type 1. For the Norwalk VLP, no binding was observed at H type 1 concentrations of 0, 0.1, and 0.25 wt%. The frequency shift then increased drastically with increasing H type 1 concentrations up to 1 wt% of H type 1, where the maximal shift was achieved. The Dijon VLP displayed a higher threshold concentration and did not show any binding to H type 1 at concentrations up to 2 wt%. Between 3 and 10 wt% of H type 1, the frequency shift increased with increasing amounts of H type 1. This suggests that the Norwalk strain requires a lower concentration of H type 1 for binding than the Dijon strain, which is consistent with the more rigid structure formed by the former strain (Figure 4). The anti-H type 1 antibody bound to the bilayer already at the lowest concentrations with increasing signals between 0.1, 1, and 10 wt%, respectively (Figure 5).

To further analyze the binding kinetics, bi-exponential functions were fit to the observed binding curves as monitored by measuring changes in resonance frequency. These functions could readily divide the observed binding processes into a rapid phase and a slower phase. The rate constants for the rapid phase at different concentrations of H type 1 for the Norwalk and Dijon VLPs are shown in Figure 6. Since the anti-H type 1 antibody binding was registered under flow conditions, binding rates were not calculated and compared to VLP binding characteristics. For both VLPs, a sharp transition from nonbinding to binding is seen at the threshold concentration of H type 1. Below this threshold, the rate constant is zero since no binding was observed. A maximum in binding rate was reached at an H type 1 concentration of 1 wt% for the Norwalk and of 5 wt% for the Dijon strain. At glycosphingolipid (GSL) concentrations above these values, the binding rate is not limited by the availability of GSLs, but only by the availability of VLPs in suspension. Under stagnant conditions, the initial binding of proteins and larger entities is typically diffusion limited, which means that the rate of binding is expected to be controlled by mass-transport rather than reaction kinetics. Indeed, using a diffusion constant of $1.1 \times 10^{-11}$ m$^2$/s for spherical particles with a radius of 19 nm and by taking the expected amount of coupled water into account (Bingen et al. 2008), the initial mass uptake ($t < 100$ s) for binding of the two VLPs to bilayers with 10 wt% of H type 1 was for both strains consistent with binding under mass-transport limited conditions (Hibbert et al. 2002). More importantly, since the two VLPs are expected to be comparable in size and hydration, this comparison revealed that the concentration of active VLPs was 1.7 times higher for the Dijon strain than for the Norwalk strain. This relative concentration difference was also in agreement with the concentrations based on determination of the total protein concentrations.

Discussion

In this study, QCM-D monitoring was used to show that norovirus VLPs specifically recognize H type 1 glycosphingolipids in supported lipid bilayers. To our knowledge, this method has not been used before to study VLP or virus adhesion to glycosphingolipids in fluid lipid bilayers. VLPs from two strains of human norovirus, representing different genogroups, were used. Both the Norwalk (GI.1) and Dijon (GII.4) VLPs bound to bilayers containing 10 wt% of H type 1, whereas no binding could be detected to bilayers containing 10 wt% of Lewis a, for any of the strains. H type 1 is a glycosphingolipid characteristic for epithelial cells of secretor positive individuals, as it is the direct product of the FUT2 gene. Lewis a, on the other hand, is a glycosphingolipid typical for epithelial cells of nonsecretors, provided they also have a functional Lewis (FUT3) gene. Structurally, the two glycosphingolipids have a common type 1 chain core structure (Galβ3GlcNAcβ3Galβ4GlcβCer) and differ only in the positioning of their fucose residues (Fuc2Gal and Fuca4GlcNAc, respectively). The glycosphingolipids used were >98% pure as determined with $^1$H-NMR (Nilsson et al. 2009) and similar also in their ceramide compositions as determined by MS (Karlsson and Larson 1981).

The specific binding to H type 1 is in agreement with previous studies of these two VLPs binding to saliva samples and to neoglycoconjugates (Marionneau et al. 2002, 2005; Rydell et al. 2009) and also with studies of many other strains of norovirus (Huang et al. 2005; Thorven et al. 2005; Le Pendu et al. 2006;
Kindberg et al. 2007; Tan, Jin, et al. 2008). For both the Norwalk and the Dijon strain, the binding specificity determined by the QCM-D assay was in full agreement with the results now presented for native VLPs binding to glycosphingolipids chromatographed on thin layer plates (Figure 1) and also in agreement with our previous studies using radiolabeled Norwalk VLPs binding to the same glycosphingolipid preparations (Nilsson et al. 2009).

The rigid immobilization of the glycosphingolipids on thin layer chromatograms thus correctly predicted the binding specificities to glycosphingolipids in a lipid bilayer, better resembling the dynamic environment of the plasma membrane. The real-time nature of the QCM-D assay furthermore enabled us to resolve the binding kinetics of the interactions. A comparison of the change in resonance frequency (cf bond mass) versus the change in the dissipation factor (cf film rigidity) during the binding of the Norwalk and Dijon VLPs revealed differences between the two strains (Figure 4). The difference in dissipation thus suggested that the Norwalk strain formed a more rigid structure together with the bilayer than the Dijon strain did. This led us to the hypothesis that the Norwalk strain was anchored to a larger number of glycosphingolipids in the bilayer, which was supported by the lower H type 1 threshold concentration observed for binding of the Norwalk strain compared to the Dijon strain when the concentration of H type 1 was lowered (Figures 5 and 6).

Multivalency is, in many cases, a hallmark of protein–carbohydrate interactions (Mammen et al. 1998). The norovirus capsid provides ideal opportunities for studies of multivalency as the VLP is composed of 180 copies of a single capsid protein (Prasad et al. 1999). Experimental support of multivalency comes from inhibition studies reporting that mM concentrations of monovalent glycans are required to inhibit binding of Norwalk VLPs to saliva (Marionneau et al. 2002). The binding of VLPs to glycosphingolipids in supported lipid bilayers should provide opportunities for optimal valencies to be formed, as the glycosphingolipids are laterally mobile. The ligand density is expected to be a key parameter for the interaction, as it affects both ligand distribution and inter-ligand distance. By varying the concentration of H type 1 between 0 and 10 wt%, we could conclude that the binding of both the Norwalk and Dijon VLPs was dependent on the density of H type 1 in the bilayer. To control for possible domain formation, Lewis a was added in these experiments so that the total proportion of glycosphingolipids in the bilayer was kept at 10 wt%. The similarity in glycan size and core structure as well as ceramide composition between H type 1 and Lewis a glycosphingolipids would suggest, but not guarantee, analogous phase properties for these structures in the lipid bilayer. In support of a multivalent interaction, both strains displayed a threshold concentration of H type 1, below which no binding was observable (Figures 5 and 6). The value of the threshold concentration was however about 10 times higher for the Dijon than for the Norwalk strain.

A similar threshold was not observed for the divalent anti-H type 1 IgG1 antibody indicating that the availability of the H epitope was linearly dependent on the H type 1 glycosphingolipid concentration even at the lowest levels. The continuous flow mode used for antibody binding avoided depletion of the antibody but the signal was instead directly dependent on the flow rate (data not shown) at the protein concentration employed.

The Norwalk strain showed a maximal shift in resonance frequency at and above 1 wt% of H type 1, whereas no such maximum was directly observed for the Dijon strain, up to 10 wt% of H type 1 (Figure 5). These differences in maximal shifts are consistent with the smaller total resonance shifts obtained for the Norwalk compared to the Dijon strain (Figure 3) and were most likely caused by depletion of the bulk concentration of the Norwalk VLP.

When the binding kinetics was analyzed by fitting bi-exponential functions to the binding curves, monitored by changes in resonance frequency, a rapid phase and a slower phase of the binding process were resolved for both VLPs (Figure 6). The transition between the two phases was possibly caused by the depletion of bulk VLPs, and thus the kinetic analysis was focused on the rapid phase. For the Norwalk strain, the kinetic analysis confirmed a threshold concentration < 1 wt% of H type 1 for the transition between nonbinding and binding, as was seen in the analysis of the total resonance frequency shifts. For the Dijon strain, however, the kinetic analysis revealed more clearly a threshold concentration between 2 and 4 wt% of H type 1 and a maximal Δf/Δt at or above 5 wt% not seen in the monitoring of total resonance shifts (Δf). Thus, even though no maximum shift in resonance frequency was directly identified for the Dijon strain, a maximum in binding velocity was clearly distinguished through this kinetic analysis.

The difference in the threshold concentration of H type 1 required for binding, identified between the Norwalk and Dijon strains, suggests that the Dijon strain must interact with a larger number of glycosphingolipids for detectable attachment to the lipid bilayer. We propose a stepwise model for the interaction between the VLPs and the glycosphingolipids in the lipid bilayer. Initially, the VLPs bind only a few glycosphingolipids, but subsequently, the binding is strengthened by additional glycosphingolipids moving into the interaction area by lateral diffusion in the bilayer. If not enough glycosphingolipids are present, then the VLP is rapidly released leading to an equilibrium coverage that is lower than the detection limit of the system. The threshold concentrations identified indicate that the monovalent interaction is too weak to provide binding in a time frame long enough for additional glycosphingolipids to reach the interaction area. Thus, the difference in the threshold concentration between the two strains suggests that the Dijon strain needs to interact with a larger number of glycosphingolipids during the initial interaction, than the Norwalk strain. The reason for this is most likely that the Dijon strain has a lower affinity for the glycosphingolipid compared to the Norwalk strain. When additional glycosphingolipids have strengthened the interaction, it is essentially irreversible. Indeed, for both strains, only a fraction of the VLPs were released by the flowing buffer.

The sharp transition from nonbinding to binding at the threshold concentration suggests the VLPs to be precise sensors of receptor density. A dependence of glycan density for binding of the Norwalk VLP has previously been suggested from ELISA experiments using immobilized H type 1 trisaccharide polyacrylamide glycoconjugates assayed at different concentrations (Marionneau et al. 2005). The existence of a threshold concentration of glycan receptors required for binding may also be of biological relevance for susceptibility to infection. Another calicivirus, the rabbit hemorrhagic disease virus (RHDV) attaches to epithelial cells from the respiratory and gastrointestinal tract through recognition of the H type 2 epitope.
(Ruvoen-Clouet et al. 2000). Under natural conditions, young rabbits are protected from infection but the susceptibility progressively increases with age. Interestingly, in histochemical studies of fixed and paraffin-embedded tracheal tissue sections, the binding of RHDV as well as the expression of H type 2 antigens in young rabbits was low, but increased with age in parallel to the susceptibility to lethal RHDV infection (Ruvoen-Clouet et al. 2000).

The clear difference in the threshold concentration of H type 1 required for binding observed between the Norwalk and the Dijon VLPs may seem surprising. However, the two strains do belong to different genogroups, a classification now based on phylogenetic analysis of the amino acid sequence of the capsid protein (Zheng et al. 2006). Currently, five genogroups are recognized, whereof genogroup I, II, and IV infect humans, whereas genogroup III and V contain bovine and murine strains, respectively. The amino acid sequences of the capsid proteins of the GI.1 Norwalk and the GI.4 Dijon strain show 43.9% identity. Cryo-EM studies of VLPs from the GI.4 Grimsby strain, with 98.7% amino acid identity to the Dijon strain, have concluded that the overall structure is similar to that of the Norwalk VLP (Chen et al. 2004). X-ray crystallographic structures of dimers of the protruding domain of the capsid protein (p-dimers) in complex with glycans have been solved for the Norwalk (Bu et al. 2008; Choi et al. 2008) and VA387 strains (Cao et al. 2007). The latter is a GI.4 strain with 99.1% capsid protein amino acid identity to the Dijon strain. These X-ray structures show that even though the viruses interact with similar glycans, the binding sites are located on different positions on the respective capsid proteins. The amino acids involved in the two binding sites are highly conserved within each respective genocluster, but not between the two genoclusters, which supports that GI.1 and GI.4 strains use distinct receptor binding sites (Choi et al. 2008; Tan, Jin, et al. 2008). The difference in the H type 1 threshold concentration identified between the Norwalk and Dijon VLPs in this study thus appears to be a reflection of the different binding sites used by the two virus strains.

In this study, we have for the first time monitored the interaction between human norovirus and glycosphingolipids incorporated in fluid supported bilayers. The results not only confirm binding data from the CBA, but also add valuable information about the multivalent nature of the protein–carbohydrate interaction. Furthermore, as effective cell culture assays are lacking for norovirus, QCM-D studies with glycosphingolipids in fluid supported lipid bilayers may be a valuable tool to assay for inhibitors of virus attachment. Such studies could include multivalent glycoconjugates and low-molecular-weight compounds, but also blocking antibodies. The study of the latter is promising as, to our knowledge, no effective assay for measuring neutralizing antibodies for noroviruses is presently available.

Material and methods

Glycosphingolipids

The glycosphingolipids used were purified from human moco- nium samples pooled from infants with the same ABO blood groups. Briefly, total neutral and acidic glycosphin- golipids were prepared as described (Karlsson 1987). In- dividual GSLs were then isolated by repeated silicic acid
were added (diluted in the CBA dilution buffer, 1:5000 for Norwalk and 1:3000 for Dijon) to the plates and incubated for 1.5 h at RT followed by the same washing procedure as in the preceding step. Secondary antibodies were added (1:1000 in the CBA dilution buffer) and washing was performed quickly once followed by three 5 min incubations in the CBA washing buffer under mild agitation. Staining was developed with Sigma Fast BCIP/NBT.

Antibody Binding Assay. The anticarbohydrate antibody assay was performed essentially in the same way as the VLP binding assay. Anti-H type 1 and Lewis antibody was diluted 1:25,000 in the CBA dilution buffer, and the secondary anti-mouse antibody was diluted 1:1000 in the same buffer. Immunostaining was developed with Sigma Fast BCIP/NBT.

Vesicle preparation
Lipid vesicles were prepared by adding 10 wt% glycosphingolipids (1 mg/mL in chloroform:methanol:water (60:35:8, by volume)) to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphorylcholine (POPC, Avanti Polar Lipids, Alabaster, ALSA) 10 mg/mL in methanol in a round-bottom flask. The solvent was evaporated under N2 to form a thin lipid film and further incubated under a slow stream of N2 at least 30 min to remove residual solvent. The lipid film was hydrated in Tris-buffer (10 mM Tris, 100 mM NaCl, pH 8.0) to a final concentration of 1 mg/mL, and mixed vigorously for 5 min. The mixture was then pressed 21 times through 30 nm polycarbonate membranes (Whatman, Maidstone, UK) using an Avanti MiniExtruder and subsequently stored at 4°C. Prior to injection in the QCM-D instrument, the glycosphingolipid-containing POPC-vesicle solutions were diluted in buffer A (10 mM CaCl2, 10 mM Tris, 200 mM NaCl, pH 8.0) to a final concentration of 0.1 mg/mL.

QCM-D
A Q-Sense E4 instrument (Q-Sense AB, Västra Frölunda, Sweden) with four parallel 40 μL sample chambers was used to conduct QCM-D measurements. SiO2-coated quartz crystals (QSX 303) were obtained from Q-Sense AB. QCM crystals were cleaned between each experiment with a 1 wt% SDS solution for ~16 h and UV-ozone treatment (atmospheric, homebuilt UV chamber) for ~60 min or O2 plasma treatment (1kW, TePla 300PC) for 10 min, and rinsed in Milli Q water before drying under a stream of N2.

The baseline was achieved in Tris-buffer after which the buffer was changed to buffer A. At least 0.5 mL of the vesicle solution was pumped through the reaction chamber at a flow rate of ~400 μL/min. When a stable bilayer was recognized, the buffer was changed back to standard Tris. VLP binding was monitored with stagnant liquid bulk. Four hundred microliters of VLPs diluted in Tris-buffer (Norwalk 10 μg/mL, Dijon 20 μg/mL) was pumped into the sample chamber. Experiments with the anti-H type 1 antibody were monitored in the flow mode at a flow rate of 10 μL/min. A cell medium containing 50 μg antibody/mL was diluted 1:10 in Tris-buffer.

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Conflict of interest statement
None declared.

Abbreviations
ALP, alkaline phosphatase; BSA, bovine serum albumin; CBA, chromatogram binding assay; D, energy dissipation; ELISA, enzyme-linked immunsorbet assay; f, frequency; FUT, fucosyltransferase gene; FucT, fucosyltransferase; G, genogroup; GSL, glycosphingolipid; Lea, Lewis a; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphorylcholine; QCM-D, quartz crystal microbalance with dissipation; RT, room temperature; TLC, thin layer chromatography; VLP, virus-like particle.

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