Human GII.4 norovirus VLP induces membrane invaginations on giant unilamellar vesicles containing secretor gene dependent α1,2-fucosylated glycosphingolipids.

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Highlights

- A human norovirus GII.4 strain recognizes secretor gene dependent glycosphingolipids
- The virus induces membrane invaginations on GUVs containing such glycosphingolipids
- These glycosphingolipids are potential receptors for human norovirus

Abstract

Norovirus is a non-enveloped virus causing acute gastroenteritis. For human norovirus, no simple cell culture system is available and consequently knowledge on cellular entry of the virus is limited. The virus binds to ABH histo-blood group glycans on glycoproteins and glycosphingolipids. Non-secretors, characterized by the lack of ABH histo-blood group glycans in the gastrointestinal tract, are resistant to most norovirus infections, suggesting that these glycans may be part of the viral receptor. Recent studies have shown that polyomavirus enters the cell via membrane invaginations induced by the multivalent binding of the virus to receptor glycosphingolipids. In this study, we have investigated whether norovirus has the ability to induce membrane invaginations on giant unilamellar vesicles (GUVs) containing purified glycosphingolipids. First, we characterized the glycosphingolipid binding pattern of VLPs from the Dijon strain (genogroup II.4), using thin-layer chromatography. The VLP recognized the ABH active glycosphingolipids H type 1, Lewis b, B type 1, A type 1 and A Lewis b, but not lactotetraosylceramide or Lewis a, typically found in non-secretors. The binding pattern to glycosphingolipids incorporated into GUVs was in full agreement with the thin-layer chromatography experiments. Upon binding to the vesicles, the VLPs formed highly mobile clusters on the surface of the GUVs. VLP containing tubular invaginations were seen on the GUVs containing glycosphingolipids recognized by the VLP. In conclusion, this study suggests that human norovirus has the ability to induce membrane curvature by binding to and clustering glycosphingolipids, which may reflect the first step in cellular entry of the virus.

Keywords: artificial membrane systems, receptor, glycobiology, histo-blood group glycans,

1. Introduction

Cell attachment and entry are essential steps of a virus infection [1]. The characterization of the host factors exploited by the virus for these steps may provide targets for antiviral drugs directed against the host instead of the pathogen [2]. For human norovirus, which causes the winter vomiting disease, the knowledge of the entry process is limited [3]. The study of this non-enveloped single stranded RNA virus has been hampered by the lack of cell culture infectivity assays [4].

Many studies of human norovirus have made use of virus-like particles (VLPs), which are formed spontaneously when the recombinant capsid protein is expressed in insect cells [5]. *In vitro* binding studies using VLPs together with immobilized neoglycoproteins and saliva samples have shown that the virus binds to glycans of the ABH histo-blood group family in a strain dependent manner [6]. For several strains, susceptibility has been shown to correlate to host blood group status, suggesting that ABH glycans may be part of the viral receptor [3]. Particularly, so-called non-secretors have been identified as resistant to norovirus infection [3]. Non-secretors constitute around 20% of the Caucasian population and are characterized by a lack of expression of ABH glycans on epithelial surfaces and in mucosal secretions. The non-secretor phenotype is caused by inactivating mutations in the fucosyltransferase 2 (*FUT2*) gene [7].

ABH glycans are found on glycoproteins and glycosphingolipids. Human norovirus VLP has been shown to adhere to both [8-11]. To our knowledge, no studies have so far addressed whether any of these glycoconjugates may be functional receptors for the virus.

Recently, a mechanism for the function of the glycosphingolipid Gb3 (Galα4Galβ4GlcCer) as receptor for the bacterial Shiga toxin was described [12]. The multivalent binding of the toxin to the receptor glycosphingolipid was demonstrated to induce the formation of tubular

invaginations on the plasma membrane of the host cell. Interestingly, the process could be reconstituted on giant unilamellar vesicles containing 1,2-dioleoylphosphatidyl choline (DOPC), cholesterol, different ceramide species of the glycosphingolipid Gb3 and a fluorescent lipid, included to visualize the membrane. Thus, tubule formation was an intrinsic property of the system and did not strictly require other cellular factors [13, 14]. The mechanisms by which membranes are bent is still not clear, but likely involves line tension, asymmetric compressive stress, a specific protein-lipid geometry, and the organization of lipids in orientation fields [12, 15]. Subsequently, the mechanism has been extended to gangliosides as receptors for polyomaviruses and cholera toxin [16, 17].

In this study we have addressed the potential of glycosphingolipids as receptors for human norovirus. The VLP studied belongs to the clinically dominating genogroup GII.4 and shows a typical secretor gene dependent binding pattern [18, 19]. It has been shown to recognize saliva samples from secretor individuals from all ABO blood groups and Lewis genotypes [19]. Neoglycoprotein binding studies have demonstrated specific binding of the VLP to α1,2-fucosylated glycans [19, 20]. These studies studies also revealed a second binding specificity to the sialylated structures including SLe^x. In addition to ABH active glycans on salivary glycoproteins, the VLP has been shown to recognize the H type 1 and Le^b epitopes on glycosphingolipids [10, 11].

In the present study we first characterized the binding pattern of the VLP to type 1 chain glycosphingolipids using a thin-layer chromatogram binding assay. Thereafter, we incorporated glycosphingolipids into GUVs and found that the Dijon VLP indeed has the ability to induce membrane invaginations upon binding to glycosphingolipids in a similar manner as Shiga toxin and polyomaviruses [12, 16].

2. Materials and methods

2.1. Glycosphingolipids

The glycosphingolipids were purified from human meconium samples pooled from individuals of the same ABO blood group or from meconium of single individuals of known ABO blood groups [21]. The individual glycosphingolipids were isolated by repeated silicic acid column chromatography of native nonacetylated and acetylated derivatives. The identity and purity of the glycosphingolipids were all analyzed as native structures by 1H-NMR spectroscopy and compared to previous characterizations by 1H-NMR and mass spectrometric analyses of permethylated and permethylated-reduced derivatives [9].

2.2. VLP

VLPs from the Dijon strain (Genbank no AAL79839) were produced by expressing the recombinant capsid protein in SF9 insect cells as previously described [19, 22]. The Dijon construct was a kind gift from Dr. E. Kohli (University of Burgundi, Dijon, France). For the GUV assay the VLPs were fluorescently labeled using a Cy3 NHS ester (PA23001, GE Healthcare, Sweden). One third of one labeling vial was dissolved in phosphate buffer (0.1 M; pH 7.5) and 500 µg of VLP was added. The reaction tube (V=400 µl) was incubated 3.5 h at RT and then over night at 4°C. Thereafter the VLPs were desalted in PBS on a Sephadex PD-10 column, final volume, 750 µl. For stability reasons BSA, final concentration 2 mg/ml, was added to the VLPs

2.3. Antibodies

The mouse anti-Lewis a IgM antibody (Seraclone, LE1 clone 78 FR 2.3) was obtained from Biotest AG (Dreieich, Germany). The ALP-conjugated anti-rabbit IgG was obtained from Sigma (St Louis, MO). The Cy3-conjugated Donkey anti mouse antibody was obtained from

Beckman Coulter (Fullerton, CA). The norovirus antiserum has been used previously in the chromatogram binding assay [10] and was obtained by immunizing rabbits with purified VLP.

2.4. Chromatogram binding assay

The chromatogram binding assay was performed as described previously [10, 23]. In brief, GSLs were applied to alumina-backed silica gel 60 HPTLC plates and chromatographed with chloroform:methanol:water (60:35:8, by volume). The plate was then dried and cut into two parts. One part was chemically stained by spraying with anisaldehyde:sulfuric acid:acetic acid (1:2:97 by volume) and heated at 180°C. The other part of the plate was plasticized with polyisobutylmethacrylate (P28, 0.3%) in hexan:diethylether (1:1 by volume) and dried. The plastic-coated plate was blocked in PBS with BSA and Tween-20 before being incubated with VLP. Antiserum against norovirus and a secondary alkaline phosphatase conjugated antibody was used for detection of bound VLPs.

2.5. Giant unilamellar vesicle assay

GUVs were grown by the electroformation technique essentially as previously described [12, 24]. The lipid composition was DOPC (64 mol %), cholesterol (30 mol %), BodipyFL-C5-HPC (1 mol %, fluorescent green) and glycosphingolipids (5 mol %). The lipid mixtures were dissolved in chloroform to a final concentration of 0.5 mg/mL. 2 x 7.5 µl of lipid mixture was evaporated on conductive indium tin oxide coated glass slides. The GUVs were grown in a sucrose solution adjusted to 300 mOsm in an alternating electric field from 20 mV to 1.1 V for 3 hours. Thereafter 10 µl of GUV solution was added to 100 µl PBS containing Cy3-labeled VLPs (20µg/mL) or the anti Lewis a antibody (diluted 1:20). The antibody was used together with a Cy3-conjugated secondary antibody diluted 1:100. Fluorescence images of GUVs were obtained at room temperature with a confocal microscope (A1R, Nikon, Japan) equipped with an oil immersion objective (60X, CFI Plan Apo VC).

3. Results and discussion

3.1. The VLP recognizes ABH active glycosphingolipids on thin-layer chromatograms

The glycosphingolipid binding pattern of the Dijon VLP was determined using a thin layer chromatogram binding assay. The structures, names and biosynthetic scheme of the glycosphingolipids used are presented in Figure 1. The VLPs recognized the α1,2-fucosylated glycosphingolipids H type 1, Le^b, A type 1, ALe^b and B type 1 (Figure 2). The synthesis of these glycosphingolipids requires a functional secretor, *FUT2*, gene and they are consequently not found in non-secretors. In contrast, the Lc_4 or Le^a glycosphingolipids typically found in non-secretors were not recognized. A glycosphingolipid fraction originating from meconium of a single blood group B individual was also assayed for VLP binding (Figure 2). This fraction contains B type 1 and BLe^b as major fucosylated glycosphingolipid components [9, 25]. The observed binding to this fraction was in agreement with binding to H type 1, B type 1, BLe^b and long chain blood group B terminated glycosphingolipids. Control plates were incubated with antibodies, but without VLP, to exclude the possibility that the anti-VLP antibodies cross-reacted with any of the glycosphingolipids. No binding was detected in this case. To conclude, the VLP recognized α1,2-fucosylated type 1 chain glycosphingolipids typically found in secretor blood group A, B, O and AB individuals. Among secretors, Lewis *(FUT3)* genotype is not expected to determine resistance to infection since structures both with the α1,4 linked fucose (Le^b and ALe^b) and without it (H type 1, A type 1 and B type 1) were recognized. The glycosphingolipid binding pattern observed was in complete agreement with earlier binding studies assaying this VLP together with neoglycoproteins and well-characterized saliva samples [19] as well as with outbreak studies and studies of antibody titers using other GII.4 norovirus strains [3].

3.2. The VLPs induce tubular invaginations upon binding to ABH active glycosphingolipids in GUVs

To verify the glycosphingolipid binding pattern in a more biological context and to investigate whether the VLP has the ability to induce membrane invaginations upon glycosphingolipid binding, VLP binding to glycosphingolipids in GUVs was tested. For these experiments, the VLP was fluorescently labeled with Cy3. In analogy with the thin-layer chromatogram binding assay experiments, the VLPs recognized GUVs containing the α1,2-fucosylated glycosphingolipids H type 1, Le^b, B type 1 and ALe^b. Upon binding to the vesicles, the VLPs formed highly mobile clusters on the surface of the GUVs. VLP containing tubular invaginations were seen on the GUVs containing binding glycosphingolipids (Figure 3, supplementary video). The invaginations contained both the fluorescent lipid, BodipyFL-C5-HPC, and the Cy3 labeled VLP. Since the GUVs were only imaged in one plane it cannot be excluded that some of the clusters were tubular invaginations pointing out of the imaged plane. The VLPs did not bind, nor induce invaginations on GUVs containing Lc_4 or Le^a. The incorporation of the Le^a glycosphingolipid into the GUVs was confirmed using the monoclonal IgM antibody together with a fluorescently labeled secondary antibody (Figure 4). The antibody bound uniformly to the vesicles and did not induce any invaginations.

In summary, the binding patterns of the Dijon VLP to glycosphingolipds separated on thin layer chromatograms and included in lipid bilayers are similar. The VLP binds to glycosphingolipids typically found in secretors irrespective of ABO blood group and Lewis genotype. Upon binding to glycosphingolipids in GUVs, the VLP has the ability to induce tubular membrane invaginations.

3.3. The multivalent presentation of the binding sites on the VLP

Compared to Shiga toxin, cholera toxin and the SV40 virus [12, 16], a lower number of tubules per GUV was observed for the norovirus VLP. This difference could be due to a difference in the localization of the binding sites. The receptor binding domains of the Shiga toxin, cholera toxin and SV40 virus are all organized into pentameric structures. For the two toxins, the B-subunit forms one single pentamer, whereas the SV40 capsid contains 72 pentamers of the VP1 protein. Despite almost no amino acid sequence homology, the three pentameric structures are very similar. On each pentamer, five binding sites are located with a distance of around 3 nm between them, even though the Shiga toxin has two additional binding sites per monomer [26]. The norovirus capsid has a T=3 icosahedral symmetry formed from 180 copies of the capsid protein organized into 90 dimers [27]. The S-domain of the capsid protein forms the shell of the particle, whereas the P-domain forms dimers extending from the shell in arch-like protrusions. Pentamers of the capsid protein are formed around the five-fold axis, but the hexamer surrounding the three-fold axis is a more abundant structure (Figure 5). The distance between the binding sites on the P-dimer of the VA387 capsid protein (99% homology to the Dijon strain) measures 2.4 nm [28]. By mapping the VA387 binding site on the crystal structure of the Norwalk VLP, based on sequence alignment (Figure 5B), the distances between the binding sites around the five- and three-fold symmetry axes may be estimated to around 6.0 and 5.4 nm respectively (Figure 5). This suggests that the binding sites on the norovirus capsid are separated by longer distances than the binding sites on the Shiga and cholera toxins as well as the SV40 virus capsid. A recent study of the binding kinetics between the Dijon VLP and small glycosphingolipid containing vesicles (radius 20-220 nm) has suggested that the VLP bound to 6-12 glycosphingolipids [11]. Noteworthy, the energy cost to deform such small vesicles is larger than the cost to deform the GUVs used in the present study.

GUV studies with Shiga toxin have demonstrated that Gb3 species with unsaturated ceramides are required for the formation of the tubular membrane invaginations [12]. For SV40, studies with GM1 neoglycolipids suggested that the acyl chain length was a more important factor for the tubule formation than the saturation degree [16]. The glycosphingolipids used in this study are purified from human meconium. The glycosphingolipids fractions are >95% pure concerning the glycans, as determined by ^{1}H-NMR but each fraction contains a mixture of ceramide species. The ceramide compositions have been determined by MS and were dominated by phytosphingosine and sphingosine with a series of hydroxylated C16:0-C24:0 fatty acids [21]. The fractions thus contain relatively long chain structures. Minor amounts of unsaturated ceramides were also detected. Importantly, the composition is similar between the different fractions.

The demonstrated ability of norovirus to induce invaginations on GUVs, in a similar manner as polyomaviruses and Shiga toxin, indicates that glycosphingolipids may be cellular receptors for human norovirus. Cell biology experiments are of course required to demonstrate the receptor function, but the formation of tubular invaginations is an initial step for other pathogens using glycosphingolipids as receptors. For the SV40 virus, the membrane invagination process was demonstrated to be involved in the infectious entry pathway [16].

Murine norovirus, which causes a systemic disease quite different from the gastroenteritis caused by human norovirus, has been suggested to use gangliosides as receptors on macrophages [29]. Interestingly, the entry of murine norovirus has been described to be independent of caveolin and clathrin, but dependent of cholesterol [30, 31]. These properties are characteristic also for the endocytosis of polyomviruses and the Shiga toxin [12, 16]. An involvement of cholesterol in human norovirus infection has been suggested by the identification of statin treatment as a risk factor for severe norovirus infection [32]. However, cholesterol may be involved in other steps of the virus infection than entry.

4. Conclusions

This study has demonstrated that a GII.4 human norovirus VLP recognizes epithelial glycosphingolipids in a secretor gene (*FUT2*) dependent manner. By binding to and clustering of glycosphingolipids incorporated into GUVs, the VLP has the ability to induce negative membrane curvature. This process may reflect the first step in cellular entry of the virus.

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7. Figure legends

Figure 1

Biosynthetic pathways for the synthesis of the histo-blood group ABH and Lewis type 1 chain glycosphingolipids. The secretor gene (*FUT2*) is underlined. The names of the structures are; Lc_4, lactotetraosylceramide; H type 1, H type 1 pentaglycosylceramide; Le^a, Lewis a pentaglycosylceramide; A type 1, A type 1 hexaglycosylceramide; B type 1, B type 1 hexaglycosylceramide; Le^b, Lewis b hexaglycosylceramide; ALe^b, A Lewis b heptaglycosylceramide; BLe^b, B Lewis b heptaglycosylceramide

Figure 2

The norovirus VLP recognizes ABH active glycosphingolipids on thin-layer chromatograms. Glycosphingolipids were chromatographed on thin-layer plates, which were either stained for glycosphingolipids using anisaldehyde (right), or plasticized and incubated with the VLP (left). Bound VLP was detected using antibodies and alkaline phosphatase (ALP) staining. The VLP recognized the secretor α1,2-fucosylated structures H type 1, Le^b, A type 1, ALe^b and B type 1, but not the Lc4 or Le^a glycosphingolipids typically found in non-secretors. The lane labeled Bmix contains partially purified meconium glycosphingolipids from a secretor blood group B individual [25]. The binding pattern observed for that lane is in agreement with binding to H type 1, B type 1, BLe^b and long chain blood group B terminated glycosphingolipids, as indicated in the figure.

Figure 3

The norovirus VLP induces tubular membrane invaginations upon binding to ABH active glycosphingolipids in GUVs. The VLP bound to and induced invaginations on GUVs

containing the α1,2-fucosylated glycosphingolipids H type 1, Le^b, ALe^b and B type 1. No VLP binding or invaginations were seen on the GUVs containing Lc_4 or Le^a. Scale bars: 2 μm.

Figure 4

The anti-Le^a antibody recognizes Le^a active GUVs but does not induce tubules on the vesicles. Scale bars: 2 μm.

Figure 5

A) The position of the GII.4 binding sites mapped on the crystal structure of the GI.1 VLP (PDB ID: 1IHM). The three- and five-fold symmetry axes are indicated and the distance between the binding sites in the different geometries are given.. The amino acids corresponding to the GII.4 VA387 fucose binding site (PDB ID: 2OBT) are shown as balls. The image was constructed using the Chimera package [33].

B) Partial alignment of the capsid protein of the Dijon strain, used in this study, and the VA387 (GII.4) and Norwalk (GI.1) strains. The amino acids interacting with the α1,2-fucose in the VA387 crystal structure are underlined. The corresponding amino acids of the Norwalk strain are in bold. The numbering refers to the amino acids of the VA387 strain.

Figure 1

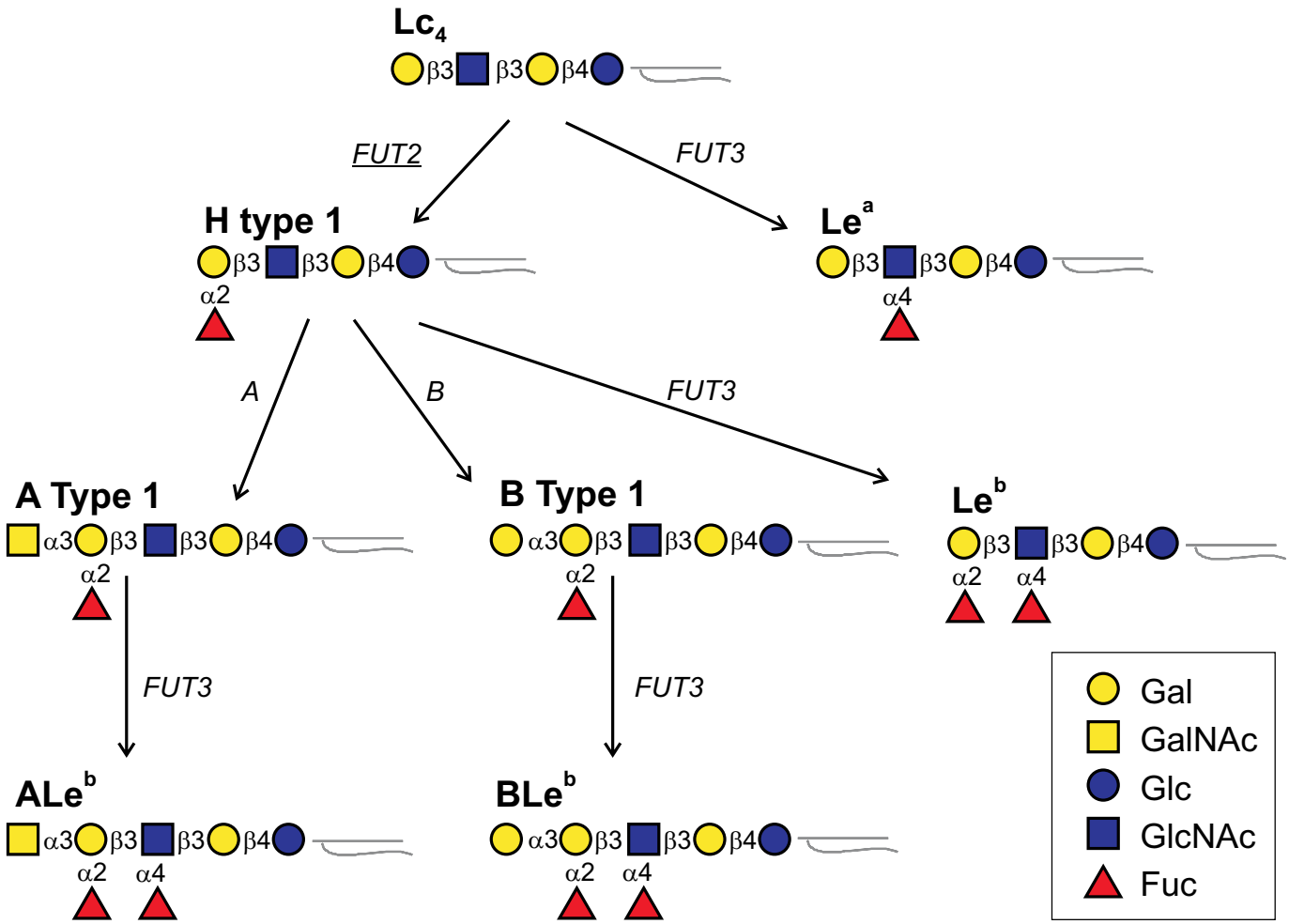

Lc4
β3 β3 β4
FUT2
FUT3
H type 1
β3 β3 β4
α2
Le^a
β3 β3 β4
α4
A
B
FUT3
A Type 1
α3 β3 β3 β4
α2
B Type 1
α3 β3 β3 β4
α2
Le^b
β3 β3 β4
α2 α4
FUT3
FUT3
ALe^b
α3 β3 β3 β4
α2 α4
BLe^b
α3 β3 β3 β4
α2 α4
Gal
GalNAc
Glc
GlcNAc
Fuc

Figure 2

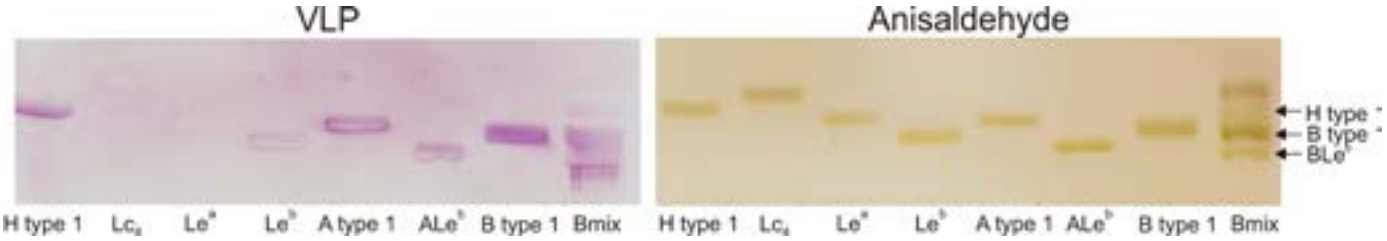

VLP
Anisaldehyde
H type 1
Lc4
Le^a
Le^b
A type 1
ALe^b
B type 1
Bmix
H type 1
Lc4
Le^a
Le^b
A type 1
ALe^b
B type 1
Bmix
H type
B type
BLe^a

Figure 3

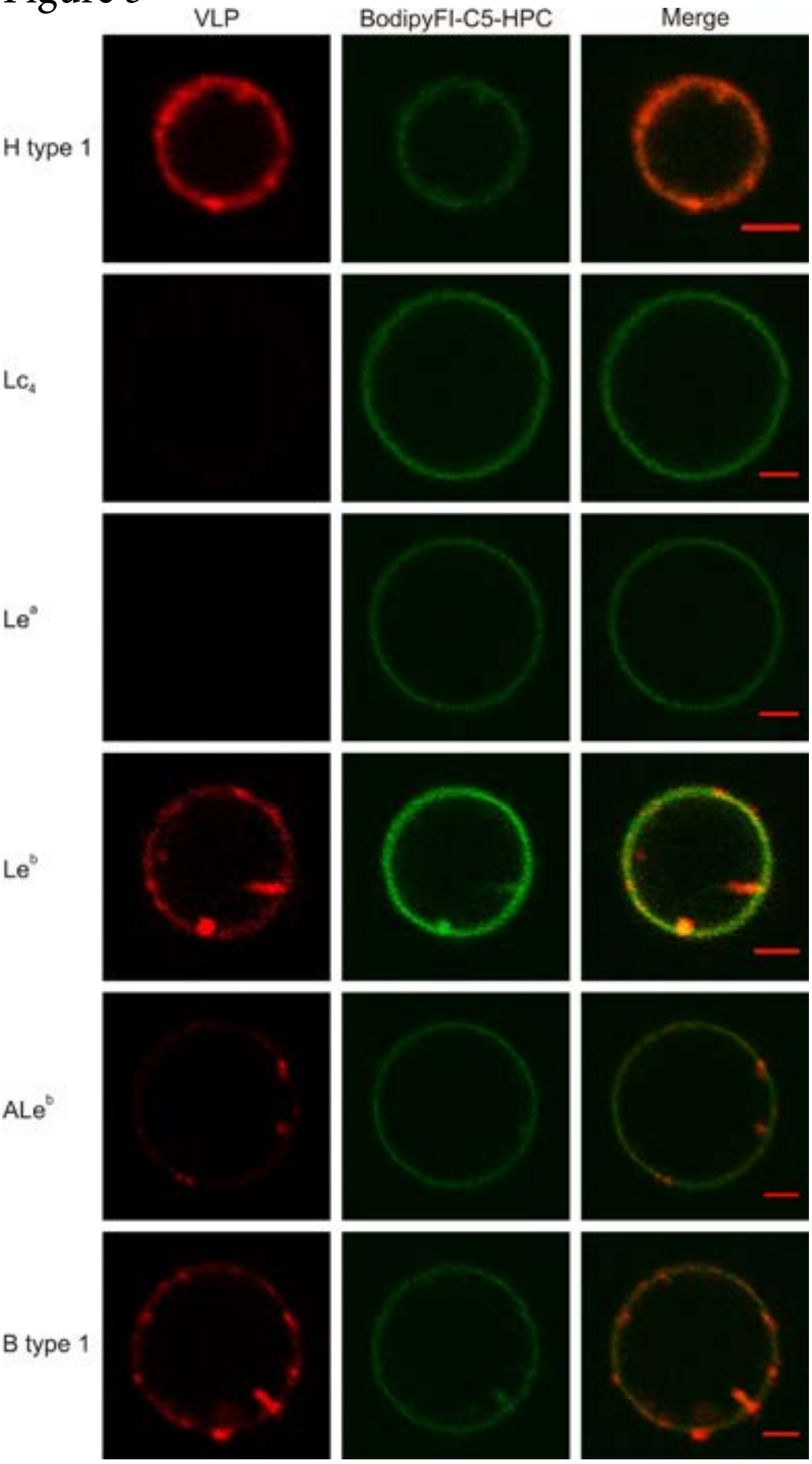


Figure 4

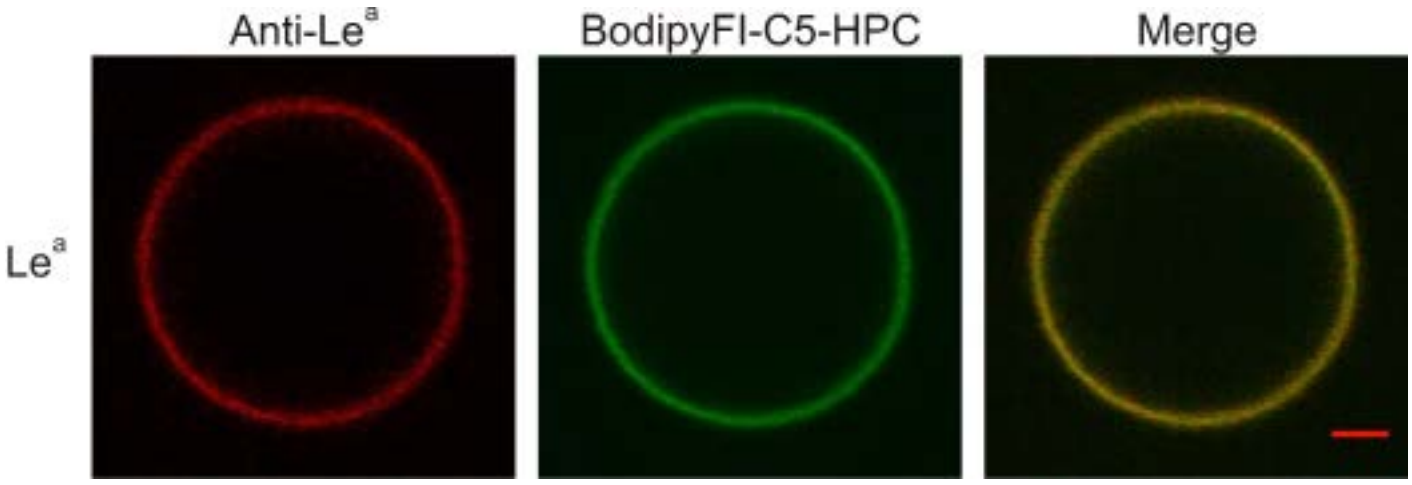
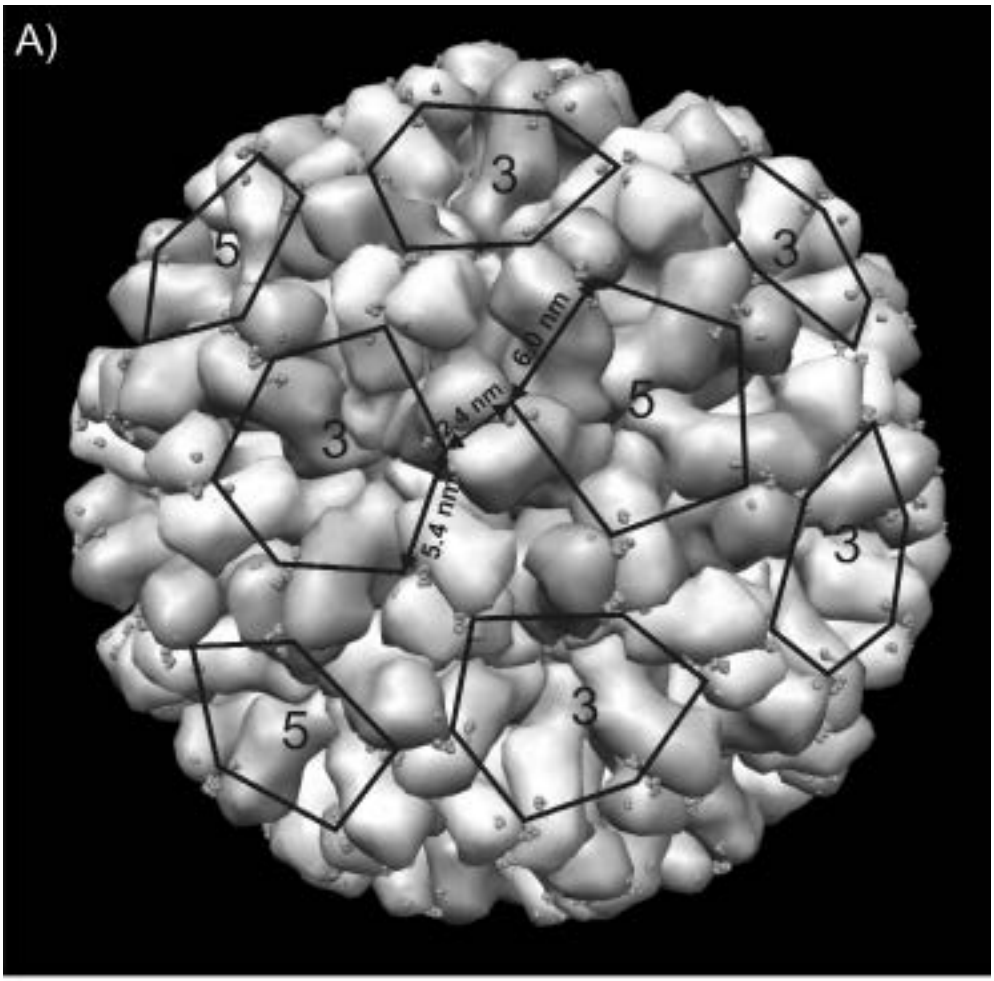


Figure 5



B)

```
                  340         349  370         379  438         447
VA387   (GII.4)   EDGSTRAHKA--DTNNDLQTG--PGCSGYPNMN
Dijon   (GII.4)   EDGSTRAHKA--DTNNDFQTG--PGCSGYPNMN
Norwalk (GI.1)    QFGHSSQTQY--GIGSGNYVG--PGPGAY---N
                  : * :   :   . ...  .*  ** ..*   *
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