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BRIEF REPORT

Comprehensive characterization of a major capsid protein derived from a documented GII.6 norovirus strain

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Abstract In this study, we successfully produced VLPs derived from full-length or chimeric VP1 of a documented GII.6 strain. Trypsin digestion of purified VLPs led to total cleavage of VP1, while the integrity of assembled VLPs was not affected. *In vitro* VLP-histo-blood group antigen (HBGA) binding and binding blockade assays indicated that trypsin digestion enhanced the binding of GII.6 VLPs to salivary HBGAs and that this binding could only be blocked by serum produced against a homologous strain. The data regarding the assembly, morphology and binding patterns of GII.6 NoV VLPs presented here might be useful for further study of GII.6 NoVs.

Keywords Noroviruses · Major capsid protein · Truncated protein · Packaging signal sequence · Blocking antibody

Noroviruses (NoVs) are the primary etiologic agents causing acute non-bacterial gastroenteritis worldwide in people of all ages [1]. NoVs are non-enveloped, single-stranded, positive-sense RNA viruses with a genome size of about 7.5-7.7 kb. They belong to the family *Caliciviridae,* which includes five genera: *Lagovirus*, *Nebovirus*, *Norovirus*, *Sapovirus* and *Vesivirus*. The genome of human NoVs consists of three open reading frames (ORFs), namely ORF1, 2 and 3, with ORF2 and 3 encoding the major capsid protein (VP1) and the minor capsid protein (VP2), respectively. VP1 can be structurally divided into a shell (S) domain and a protruding (P) domain, with the latter being further divided into P1 and P2 domains [2, 3]. Based on the amino acid sequence of VP1, NoVs can be divided at least into six genogroups, namely GI through to GVI, with members of GI, GII and GIV infecting humans [4, 5]. NoVs in GI and GII can be further divided into more than 30 genotypes.

Currently, there are no efficient *in vitro* culture methods or appropriate small-animal models for human NoVs [6, 7]. The discovery that expression of VP1 leads to self assembly of virus-like particles (VLPs) with morphology and antigenicity similar to those of true virions and the observation that NoV infections are correlated with secretor status has greatly advanced our understanding of this group of viruses [8, 9]. However, human challenge studies have demonstrated that other factors might also be involved, as secretor-negative individuals can be infected by non-GI.1 and GII.4 NoVs [10]. Histo-blood group antigens (HBGAs) are presumed to be receptors or co-receptors for NoVs, and most VLPs derived from VP1 of different genotypes exhibit certain binding patterns, suggesting that binding to HBGAs might be a necessary step for NoV infection [11].

The GII.6 NoV infection rate has been maintained at a constant low level in most countries, while it predominated in Japan from 2008 to 2009 [12]. A recent epidemiological study indicated that the GII.6 NoV infection rate was second to GII.4 NoV, with GII.3 NoV being the third most frequently isolated [13]. The close relationship of the GII.6 NoV and GII.3 NoV sequences and the fact that protein

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structure simulation indicates that both strains share a surface-exposed loop suggests they might share a common mechanism of infection, that differs from that of GII.4 NoV [14, 15]. Considering that the rate of GII.3 NoV infection ranks second or third overall, a comparative study of GII.6 NoV will provide important data about how infectivity is determined and what role HBGAs play in infection [16].

In this study, we codon-optimized and then expressed the major capsid protein (VP1) gene derived from a documented GII.6 NoV strain by using a recombinant baculovirus expression system. The assembly of VLPs with full-length and chimeric VP1 and their ability to bind to salivary HBGAs before and after trypsin digestion were evaluated. An *in vitro* VLP-salivary HBGA binding blockade assay was performed to determine cross-blocking effects of hyperimmune serum against different genotypes.

The VP1 amino acid sequence of a documented GII.6 strain (GenBank accession number AB818400) was used for codon optimization and synthesis. The optimized sequence was inserted into transfer vector pBacPAK9 with a BamHI and NotI restriction enzyme site at its 5′ and 3′ end, respectively. For the expression of chimeric VP1, the DNA binding sequence (MSTTAKRKKRKL) derived from human papillomavirus 16 was codon-optimized and used to replace the N-terminal 26 amino acids of VP1 [17]. The generation of recombinant baculovirus was performed as described previously [18]. Expressed VP1 and chimeric VP1 were purified by cesium chloride gradient density centrifugation. The purity of purified VP1 and chimeric VP1 and the proper assembly of VLPs was determined by SDS-PAGE and electron microscopy (EM), respectively.

For digestion analysis, CsCl-purified GII.6 NoV VLPs (4 μg) were digested with cell-culture-grade trypsin at a final concentration of 200 μg/ml (a concentration at which complete cleavage was achieved) in phosphate-buffered saline (PBS; 0.01 M **??xxx??**, pH 7.2) containing 0.05% Tween-20 (PBS-T). The digestion was performed at 37 °C for 30 min. GII.6 NoV VLPs that were not treated with trypsin were used as a control. The digested products were analyzed by SDS-PAGE. The integrity of the VLPs before and after treatment was analyzed by EM. To determine the molecular size of the generated fragments after digestion, the corresponding bands in SDS-PAGE gels without staining were cut out and processed for molecular weight (MW) analysis. The purified protein sample was subjected to 5800 MALDI-TOF/TOF (AB SCIEX) analysis, and the raw data were analyzed using 4000 Series Explorer V3.5 software.

The ability of purified and trypsin-digested GII.6 VLPs to bind to salivary HBGAs was analyzed. Saliva samples were collected from blood type A, B, AB and O individuals. The presence of HBGA antigens in the saliva samples was demonstrated in our previous study [18]. GII.4 (KF306214) VLPs produced in Sf9 cells using a recombinant baculovirus expression system were used as reference. The procedure for the *in vitro* salivary HBGAs-VLP binding assay was the same as described previously with minor modifications [19]. All VLPs were used at 0.5 μg/ml. PBS-T containing 1% BSA was used as a negative control. The procedure for preparation of rabbit anti-GII.6 VLP hyperimmune serum was described previously [20]. Rabbit sera were collected from the cervical artery under anesthesia.

The *in vitro* salivary HBGA-VLP binding blockade assay was performed in basically the same way as the *in vitro* VLP-salivary HBGAs binding assay except that the GII.6 and GII.4 VLPs (1 μg/ml) were pre-incubated with rabbit anti-genotype-specific hyperimmune sera (GI.2, GII.3, GII.4, GII.6, GII.7 and GII.17, diluted at 1:400) or rabbit serum at 37 °C for 30 min before being transferred to wells coated with blood type B saliva samples [21]. Wells (in duplicate) to which only PBS-T was added were selected as positive controls. The blocking index was calculated in % as (mean OD without sera–mean OD with sera)/mean OD without sera × 100%.

To improve protein expression levels, the GII.6 NoV VP1 amino acid sequence was used for codon optimization based on codon usage frequency of *Spodoptera (S.) frugiperda* cells. For chimeric VP1 expression, the DNA-binding sequence derived from the HPV 16 L1 protein was optimized and fused to the N-terminus of truncated GII.6 NoV VP1 with the N-terminal 26 amino acids deleted. Infection of Sf9 cells with recombinant baculovirus encoding GII.6-VP1 and GII.6-VP1-L1BS led to high level expression of both proteins as determined by SDS-PAGE after purification. The purified proteins were negatively stained for EM observation at a concentration of 200 μg/ml, and VLPs were observed for both proteins (Fig.1 A and C). VLPs derived from both proteins exhibited no significant difference in size or morphology. It has been shown that the N-terminal sequence is not essential for assembly of VP1 into VLPs [22]. In this study, we demonstrated that NoV VP1 can tolerate heterologous sequences, and this characteristic might be useful for other studies, such as DNA packaging studies.

Sequence alignment of VP1 from different genotypes indicated a short sequence insertion in the P2 domain of VP1 of GII.3 and GII.6 NoVs. Homology modeling indicated that the inserted sequence formed a surface-exposed loop that was susceptible to trypsin digestion [14]. To test if GII.6 NoV VLPs exhibit susceptibility and cleavage patterns similar to that of GII.3 NoV VLPs, trypsin digestion was performed. As shown in Fig.1D, trypsin digestion produced two bands with a molecular weight (MW) of approximately 26 and 31 kDa, respectively. Using the cleavage patterns of GII.3 NoV VLPs as a reference, the 31-kDa band should be the N-terminal half of VP1, and the 26-kDa

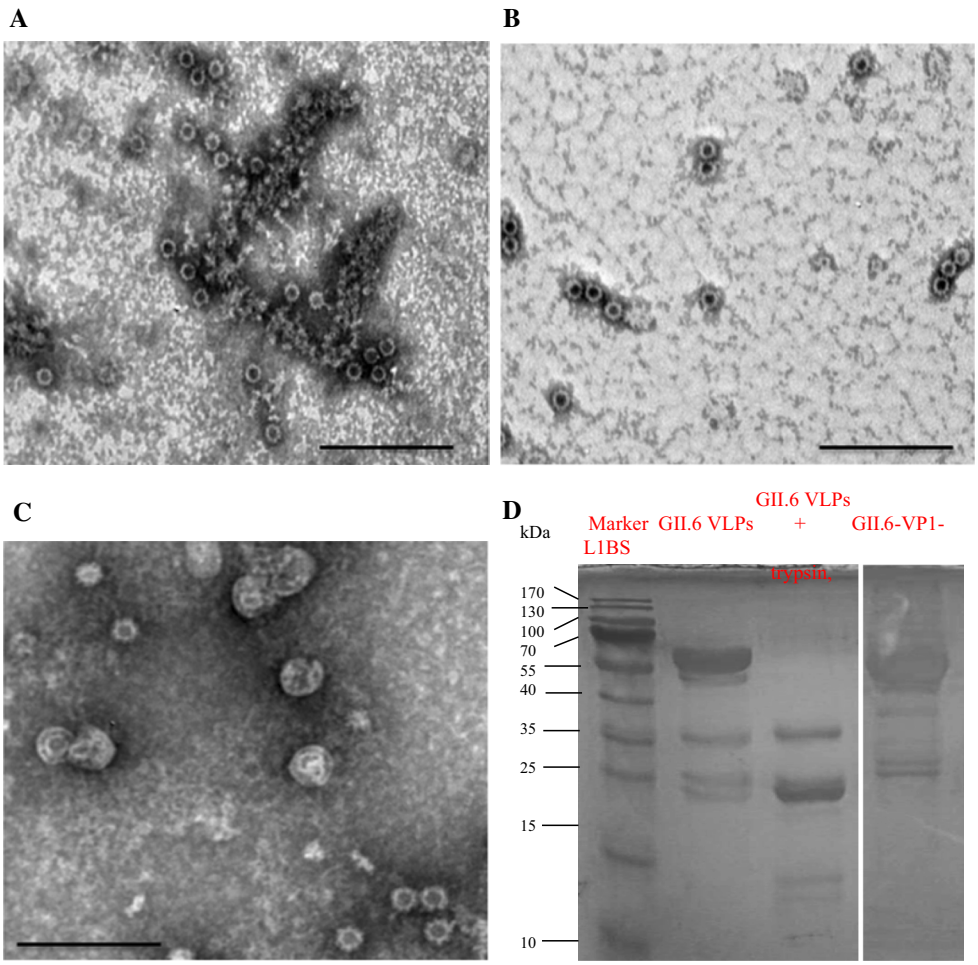

Fig. 1 EM and SDS-PAGE analysis of purified GII.6 full-length and chimeric VP1 with or without trypsin digestion. Expression of GII.6 full-length (A) and chimeric (C) VP1 led to successful assembly into VLPs. Digestion of GII.6 VLPs by trypsin led to total cleavage of VP1 (D), but intact VLPs were still observed (B). Magnification ratio, 70,000 x for A and B, and 100,000 x for C. Scale bar, 200 nm. The marker used was a prestained protein marker

band should be the C-terminal half. To predict the N-terminal amino acid sequence of the 26-kDa fragment, the band was subjected to MW determination by MS analysis. The 26-kDa band gave a MW of 26,118.2520 (m/z) by MS, and the predicted bands corresponded to residues 308 to 543, with the N-terminal four amino acids removed by cleavage (Fig. 2). To determine the actual N-terminal residues of the 26-kDa band, N-terminal amino acid sequencing was attempted, but for unknown reasons, the sequencing result was inconclusive (data not shown).

To determine if the cleavage affects the integrity of assembled VLPs, GII.6 VLPs with and without trypsin digestion were examined using EM. As shown in Fig.1D, SDS-PAGE indicated that VP1 was completely cleaved in trypsin-digested VLPs, while EM analysis (Fig.1B) indicated the presence of intact VLPs even after cleavage. Only limited numbers of VLPs were observed in EM pictures of both treated and untreated VLPs, but considering that VP1 was completely cleaved in the treated VLPs, our results still give strong evidence that trypsin digestion or trypsin cleavage at the surfaced-exposed loop did not affect the morphology of the assembled VLPs. The maintenance of morphology after cleavage might be due to the intrinsic flexibility of the assembled VLPs caused by intramolecular interactions such as the presence of a disulfide bond.

The ability of GII.6 NoV VLPs before and after trypsin cleavage to bind to salivary HBGAs derived from blood type A, B, AB and O individuals was determined using an *in vitro* salivary HBGA-VLP binding assay. GII.6 VLPs exhibited binding activity against all saliva samples tested, with highest binding capacity against blood type A saliva samples and lowest to blood type O saliva samples. Compared with untreated VLPs, trypsin digestion enhanced the binding of GII.6 VLPs to salivary HBGAs derived from individuals of all blood types (Fig. 3). The signal values increased less than 50% for most saliva samples after trypsin digestion, while it increased by 5-fold for a saliva sample derived from a blood type O individual.

Based on the above salivary HBGA-VLP binding results, a blood type B saliva sample was used. To test if trypsin cleavage is associated with a newly formed binding interface, the difference in binding capacity between GII.6 VLPs with and without trypsin digestion was also tested. Our results showed that the binding of GII.6 VLPs against

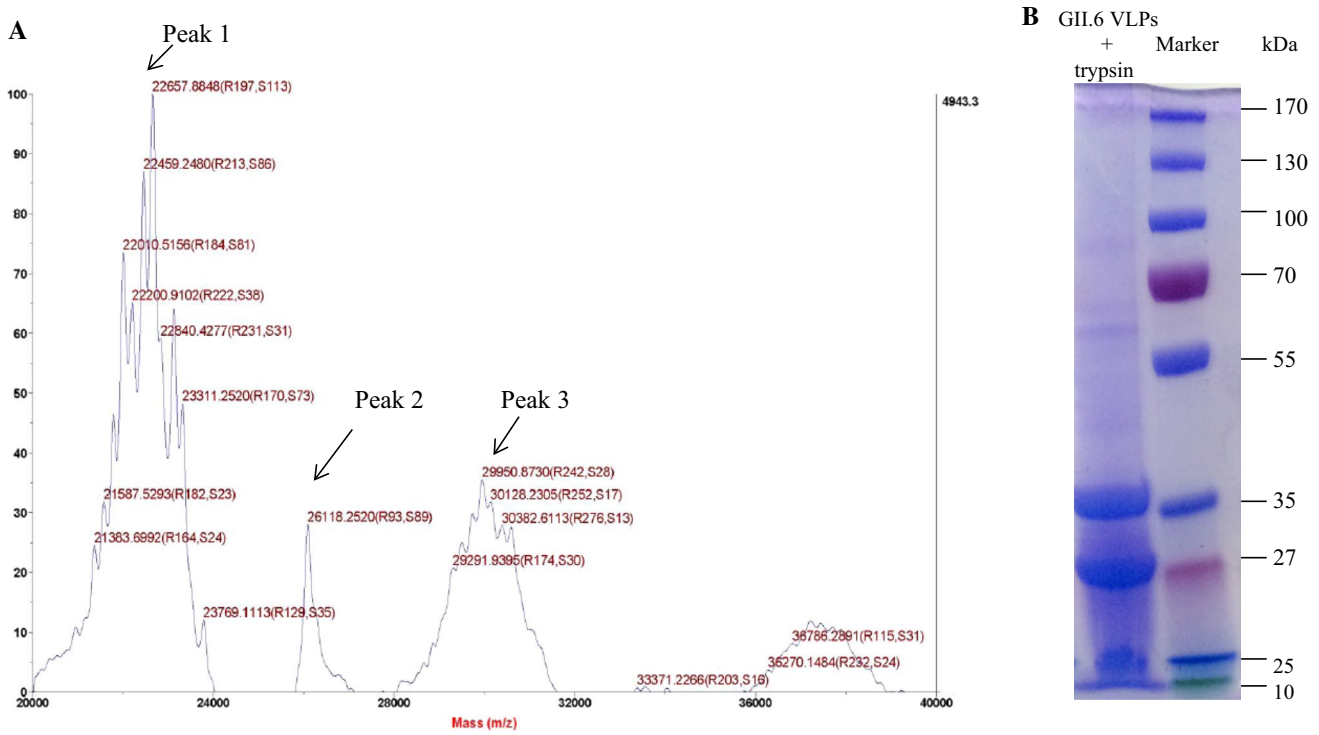

Fig. 2 Mass spectrometry of fragments produced by trypsin cleavage of GII.6 VLPs. GII.6 VLPs (200 μg) were diluted with PBS-T to a concentration of 0.5 mg/ml, and trypsin was added to a final concentration of 400 μg/ml. The mixture was incubated at 37 °C for 30 min and then analyzed by SDS-PAGE for cleavage completion. The unstained 26-kDa band was cut out by comparison with a stained gel (B) for molecular weight determination (A). The presence of multiple peaks might be caused by contamination of the 31-kDa band with trypsin-derived materials. Peak 3 most probably reflects the MW of the 31-kDa band

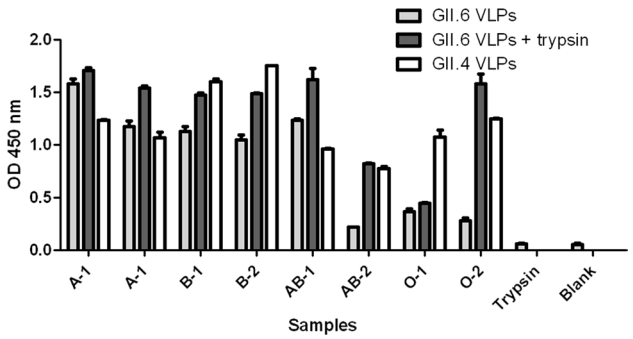

Fig. 3 Cleavage of GII.6 VLPs by trypsin promotes its binding to salivary HBGAs. GII.6 and GII.4 VLPs bound to salivary HBGAs from blood type A, B, AB and O individuals. Trypsin digestion of GII.6 VLPs promoted its binding to all saliva samples. GII.6 VLPs (4 μg) were digested with trypsin at 200 μg/ml in phosphate-buffered saline containing 0.05% Tween-20 before being added to wells at a final concentration of 0.5 μg/ml. GII.6 and GII.4 VLPs without treatment were diluted in PBS-T to a final concentration of 0.5 μg/ml and added directly to saliva-precoated wells. PBS-T buffer with or without trypsin was used as a negative control

salivary HBGAs could be blocked by rabbit anti-GII.6-VLP specific hyperimmune serum, but not by anti-VLP sera against other genotypes (GI.2, GII.3, GII.4, GII.7 and GII.17). The genotype-specific blocking effect was observed even after trypsin digestion of GII.6 VLPs, suggesting the conservation of binding epitopes before and after cleavage.

In this study, we successfully expressed the VP1 of a documented GII.6 NoV strain. It has been shown that GII.3 VLPs are susceptible to trypsin cleavage in the surface-exposed loop region [14]. Treatment of GII.3 VLPs with trypsin led to generation of two bands with a molecular weight of 26 and 31 kDa, respectively. As expected, GII.6 VLPs were also susceptible to trypsin digestion, and cleavage generated two bands with sizes similar to those of GII.3 VLPs. To determine the exact sequence range, we first performed MS analysis and then predicted the possible N-terminal residue of the 26 kDa band by comparing it with that of a previously reported GII.3 NoV. We also tried to determine the actual N-terminal residue of the 26-kDa band by N-terminal amino acid sequencing, but without success. Based on our data and literature reports, GII.3 and GII.6 VP1 were possibly cleaved at similar sites. Trypsin cleavage did not affect the morphology of GII.3 NoV VLPs, and this phenomenon was also observed for GII.6 NoV VLPs. However, it should be noted that after cleavage, GII.6 NoV VLPs tended to disassemble to a certain degree. The observed disassembly of VLPs might be caused by

other factors; for example, the time period was generally greater than 12 hours between negative staining of samples and EM observation. In fact, our recent study demonstrated total disassembly of GII.3 VLPs after trypsin cleavage [23]. The differences might be related to the experimental procedures used.

An *in vitro* salivary HBGA-VLP binding assay indicated that GII.6 VLPs bound to saliva samples from all blood types, which is consistent with a previous report [18]. An *in vitro* salivary HBGA-VLP binding assay with digested GII.6 VLPs indicated that cleavage enhanced the binding to salivary HBGAs. The observed enhanced binding was possibly due to polymer formation by the cleaved fragments. Our hypothesis is based on several observations. First, the limited number of VLPs observed by EM suggested the presence of a large proportion of free VP1 monomers or dimers and thus the formation of P dimers, small P particles or P-particle-like complexes after cleavage [24]. Second, VLPs prepared by PEG 6000 precipitation exhibited no binding enhancement after trypsin digestion despite the fact that SDS-PAGE indicated total cleavage of VP1 (data not shown). Compared with CsCl-purified VLPs, PEG-6000-purified VLPs should contain less disassembled VP1, since harsh conditions were avoided. Third, a time-course study of trypsin-digested GII.6 VLPs indicated that increasing the digestion time did not significantly increase the ability of VLPs to bind to salivary HBGAs (data not shown). A reasonable explanation for this could be that free VP1 monomers or dimers in purified VLPs were rapidly cleaved and that this was reflected in the rapid appearance of peak binding signals. The cleaved full particles were stable and might undergo disassembly slowly, as indicated by a gradual increase in the binding signal. If our hypothesis is correct, and if we presume that VLPs exhibit biochemical characteristics similar to those of true virions, cleavage might have an important biological role in NoV infection, as has been observed for astroviruses and HPV [25, 26].

In summary, our data indicate that the HBGA binding spectrum is not associated with the epidemic status and that there are other unknown susceptibility determinants. The tolerability of heterologous sequences in the N-terminus of VP1 suggests that the N-terminal sequence is not essential for VLP assembly. If the sensitivity of GII.6 VLPs to trypsin digestion applies to native virions, trypsin cleavage might play an important role. The chimeric VP1 constructed in this study can be used to package foreign sequences, such as a plasmid encoding reporter genes for receptor binding and VLP entry studies and NoV VP1 genes for vaccine development (subunit vaccine combined with nucleic acid vaccine). These studies will certainly facilitate the discovery of cellular components mediating NoV entry and the development of combined vaccines.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval Animal experiments were performed in accordance with the guidelines of the Chinese Council on Animal Care. The research protocol was approved by the Animal Care and Use Committee of the Wuhan Institute of Biological Products (WIBP).

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