Assembly of homogeneous norovirus-like particles accomplished by amino acid substitution

Yuichi Someya,1 Haruko Shirato,1 Kazuya Hasegawa,2 Takashi Kumasaka2 and Naokazu Takeda1†

1Department of Virology II, National Institute of Infectious Diseases, Japan
2Japan Synchrotron Radiation Research Institute, Japan

Infection of insect cells with recombinant baculoviruses carrying the VP1 gene from Chiba strain norovirus resulted in the production of 57 and 50 kDa proteins, and the assembly of a smaller, 23 nm form of the virus-like particles (VLPs), together with the normal, 38 nm form of the VLPs. The N-terminal residues of the 57 and 50 kDa proteins were Ala4 and Thr45, respectively. When the tripeptide Leu43-Ala44-Thr45 was changed to Ala-Pro-Val, only 38 nm VLPs were assembled. The 38 nm VLPs showed essentially the same pattern of carbohydrate binding as the 23 nm VLPs, despite the significant difference in the degree of Lewis b antigen binding.

Noroviruses are major causative pathogens of non-bacterial acute gastroenteritis in humans. Increasing numbers of strains are being isolated worldwide (Glass et al., 2009; Patel et al., 2009). Based on their sequence diversity, human noroviruses are divided into two major genogroups, genogroups I (GI) and II (GII), and nearly 40 genotypes have been identified in these genogroups (Okada et al., 2005). A wide variety of antigenicity, which is nearly parallel to genetic variety, has complicated the development of vaccines that are effective for all genotypes. In contrast, the high specificity of antibodies to a specific norovirus, which were raised against recombinant virus-like particles (VLPs) expressed in insect cells, enabled us to develop a serotype-specific antigen ELISA (Kobayashi et al., 2000a, b, c).

We have previously reported a preparation of VLPs of Chiba strain norovirus (GI, genotype 4) (Kobayashi et al., 2000a). Chiba VLPs were used to prepare rabbit antisera (Kobayashi et al., 2000a) and to examine their interaction with histo-blood group antigens (Shirato et al., 2008). As reported in Kobayashi et al. (2000a), when recombinant baculoviruses encoding the wild-type Chiba ORF2 gene infected Tn5 insect cells, particles smaller than naturally occurring norovirus particles (23 nm species) were generated, together with normal-size (38 nm species) particles (Fig. 1a). SDS-PAGE analysis of purified wild-type VLPs revealed that a truncated form of the VP1 protein (apparent Mr, 50 kDa) was present, as well as intact VP1 proteins (57 kDa) (Fig. 1c, lane 4); this is similar to a previously reported phenomenon observed in Norwalk strain norovirus, the prototype norovirus (White et al., 1997). It was suggested that each of the smaller VLPs consisted of both 50 and 57 kDa VP1 proteins, since the number of truncated proteins was almost equal to that of the intact proteins (White et al., 1997). This led us to raise the question of whether the heterogeneous mixture of VLPs reflected all properties of native norovirus virions.

To investigate where a VP1 protein is truncated to yield a 50 kDa protein, VLPs were subjected to mass spectrometric analysis. As a result, four major peaks were identified. Two peaks of 57.4 and 56.9 kDa corresponded to a 57 kDa band, and two peaks of 53.6 and 53.1 kDa corresponded to a 50 kDa band. According to the results of amino acid sequencing of bands excised from an SDS-PAGE gel, the 57 kDa proteins were a mixture of proteins whose N termini started with Ala4, Ser5 or Ala12, with the Ala4 species being the most abundant. Although the N-terminal amino acids deduced from the nucleotide sequence are Met1-Met2-Met3-Ala4-Ser5, this result suggests that the three consecutive N-terminal methionine residues were eliminated post-translationally in insect cells. On the other hand, almost all of the 50 kDa proteins were shown to have the Thr45 residue in their N termini, indicating that the peptide bond between Ala44 and Thr45 was susceptible to proteolytic agents in insect cells. The Thr45 residue is conserved in VP1 proteins from GI, but it is replaced with valine or leucine in GII. The Leu-Pro or Val-Pro dipeptide appears earlier in the sequence than Thr45 in GI VP1 proteins and, in contrast, the Ala-Pro dipeptide is conserved in GII VP1 proteins. It is possible that the generation of smaller VLPs from Chiba strain VP1 proteins can be attributed to the amino acid sequence around the Thr45 residue. It has been observed that expression of the VP1 genes from some GII strains resulted in the
production of two major protein bands and two kinds of VLPs, as observed for the wild-type Chiba VP1. However, it is clear that the GII VP1 protein of lower molecular mass migrates much faster in SDS-PAGE gels than the protein predicted to be produced if it were truncated at the position corresponding to the cleavage site in Chiba VP1 protein (data not shown), although detailed analyses for GII VP1 have not yet been done. It is suggested that GII VP1 is truncated at a different site. Nevertheless, we thought that conversion from the original sequence (Leu43-Ala44-Thr45) of Chiba VP1 to the GII-type sequence (Ala-Pro-Val) was a possible genetic approach that we could attempt.

The EcoRI–PstI fragment including the entire ORF2 and ORF3 genes of the Chiba strain was excised from pUCCVORF2,3, in which the ORF2 and ORF3 genes had been cloned by using primers SRSV-D5 and CV-U5 (Supplementary Table S1, available in JGV Online) from Chiba strain cDNA (Someya et al., 2000). The fragment was transferred to the pORB baculovirus transfer vector.

Fig. 1. Electron micrographs and SDS-PAGE analysis of VLPs. (a, b) Electron micrographs of the wild-type VLPs (a) and the triple-mutant VLPs (b). Bar, 100 nm. (c) SDS-PAGE analysis of VLPs. Prior to electrophoresis, samples (5 µg of protein) mixed with sample buffer were heat denatured (+) or not heated (−). Gels were stained with Coomassie brilliant blue. (d, e) Western blot analysis. VLPs (6 µg of protein) were separated by SDS-PAGE and then electroblotted onto a PVDF membrane, followed by detection with rabbit antiserum raised against the wild-type VLPs (d) or with rat antiserum raised against the triple-mutant VLPs (e).
(Orbigen), with the resultant plasmid being designated pORBCVORF2,3. Recombinant baculoviruses were collected after transfection of S9 cells (Invitrogen) with pORBCVORF2,3 and commercially available baculovirus DNA (Orbigen or Sigma-Aldrich), and were used for the infection of HighFive (Tn5) cells (Invitrogen). VLPs were collected from insect media, isolated by passing them through a caesium chloride density gradient and resuspended in unsupplemented Grace’s insect media (Invitrogen). Recombinant baculovirus derived from wild-type pORBCVORF2,3 resulted in the production of smaller VLPs concomitantly with the production of normal-size VLPs, similarly to the case of the pVL-derived baculovirus shown in Fig. 1(a). The various GII-type mutations were then introduced into pORBCVORF2,3. Mutagenesis was performed by PCR using each set of two oligonucleotides (PSF and PSR primers) listed in Supplementary Table S1. Mutations were first detected by restriction analysis and then verified by DNA sequencing. Since the Ala44–Thr45 peptide bond seems susceptible to protease(s) in insect cells, we tested the introduction of the various GII-type mutations around the Thr45 residue. Neither the A44P single mutant nor the L43A, A44P double mutant blocked the generation of the smaller protein band. Although the T45V single mutation led to the production of the 57 kDa band only, no VLPs were formed in insect cells (data not shown). Normal-size particles were successfully generated by the L43A, A44P, T45V triple-mutant (Fig. 1b), and mutated VP1 proteins gave an apparently single band of 57 kDa on an SDS-PAGE gel (Fig. 1c, lane 2). When VLP samples were not heated prior to being subjected to SDS-PAGE, the triple-mutant VLPs gave a single band of approximately 100 kDa (Fig. 1c, lane 1). In contrast, the wild-type VLPs gave three bands of approximately 100, 85 and 70 kDa (Fig. 1c, lane 3). These results suggested the formation of tightly bound dimers. The three protein bands from the wild-type VLPs probably corresponded to 57 kDa homodimers, 57–50 kDa heterodimers and 50 kDa homodimers, respectively. The electrophoretic mobility of each dimer was much greater than that estimated from the calculated molecular mass. This result suggests that the dimers are globular in shape in the gel, as it is well known that differences in protein shape affect electrophoretic mobility (Robyt & White, 1987).

An antigen-detection ELISA kit that contains anti-Chiba strain antibodies is commercially available in Japan (Kamata et al., 2005). These antibodies, raised against the wild-type VLPs in rabbit, reacted not only with both the 57 and 50 kDa bands of the wild-type VP1 proteins, but also with the 57 kDa band of the triple-mutant VP1 proteins (Fig. 1d). Conversely, rat antisera raised against the triple-mutant VLPs reacted with both the wild-type (57 and 50 kDa) and the triple-mutant (57 kDa) VP1 proteins (Fig. 1e). These results indicated that the 50 kDa proteins retained the region where rat antisera against the triple-mutant VLPs reacted, and that the antigenicity was preserved despite the introduction of the triple-mutation.

Shirato et al. (2008) have previously shown that the wild-type VLPs from the Chiba strain bound to synthetic type A carbohydrates of histo-blood group antigens as well as Lewis a and Lewis b carbohydrates. Here we examined whether or not the triple-mutant VLPs have the same characteristics as the wild-type VLPs. ELISA-based binding assays using biotinylated multivalent carbohydrate reagents (Glycotech) were performed as described previously (Shirato et al., 2008). Briefly, multivalent carbohydrate–biotin reagents (Glycotech) were immobilized onto 96-well streptavidin-coated plates (Thermo Electron Corporation), and 100 µL of VLPs (1 µg mL⁻¹) were added to each well, followed by immunochemical detection with rabbit anti-Chiba VLP antiserum and HRP-conjugated anti-rabbit IgG, as the primary and secondary antibodies, respectively. Finally, O-phenylenediamine was added to visualize the carbohydrate binding of VLPs, and A₄₉₂ was measured. As shown in Fig. 2, the triple-mutant VLPs bound to the type A, Lewis a and Lewis b antigens, showing essentially the same pattern as the wild-type VLPs. The binding of the triple-mutant VLPs to the Lewis b carbohydrates was weaker than for the wild-type VLPs. It is notable that the wild-type VLPs are a mixture of 38 nm VLPs and smaller 23 nm VLPs. The results of the carbohydrate-binding experiment suggest that Lewis b antigens bind to the 23 nm VLPs more strongly. It is possible that these two VLP species differ from each other in terms of the number of VP1 protein molecules, as predicted by White et al. (1997); that is, the 38 nm VLP was formed by 90 dimers (180 molecules) of the VP1 protein with T=3 symmetry, whereas the 23 nm VLP was formed by 30 dimers (60 molecules) with T=1 symmetry. This suggests that the local structure of the carbohydrate binding site(s) differs slightly between the two VLP species. Since the numbers of molecules included are different in the two VLP forms, the size of the cavity between the protruding (P) domains from two neighbouring molecules might also be different. The weaker binding of the triple-mutant VLPs (and possibly the 38 nm wild-type VLPs) to Lewis b antigen might be caused by a difference in the molecular architecture of the VLPs.

The Leu43-Ala44-Thr45 tripeptide (Val-Ala-Thr in Norwalk virus VP1) is located in the N-terminal arm region preceding the shell (S) domain in the crystal structure (Prasad et al., 1999). It exists inside the VLP and is probably involved in the intermolecular interaction. The crystal structure showed that the tripeptide in the A subunit was located in the vicinity of the tripeptide in the C subunit. This suggests the possibility that the lack of the N-terminal 44 aa affects the interaction between the S domains of two monomers and hence the molecular architecture of the VLPs. It is conceivable that some change in the S domain may affect the configuration of the P domain, which is thought to be responsible for the carbohydrate interaction.

Considering our results described in this manuscript, we observe that whether or not the exogenously expressed...
VLPs in insect cells are homogeneous in size is important, as the profile of carbohydrate binding for each strain of noroviruses might be misleading otherwise. It is worthy of note that the introduction of the appropriate mutation(s) enables homogeneous expression of VLPs whose size is identical to that of naturally occurring norovirus virions.

Acknowledgements

This work was supported partly by Grants-in-Aid from the Ministry of Health, Labour and Welfare, Japan. We are grateful to Dr Ken’ichi Hagiwara (Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Japan) for amino acid sequencing, to Dr Michiyo Kataoka (Department of Pathology, National Institute of Infectious Diseases, Japan) for electron microscopic analysis, and to Dr Tatsuya Yamamoto (Biomedical Science Laboratory, RIKEN Spring-8 Center, RIKEN, Japan) for mass spectrometric analysis.

References


