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Unusual self-assembly properties of Norovirus Newbury2 virus-like particles ☆

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ABSTRACT

In the Caliciviridae family of nonenveloped, positive-stranded RNA viruses, Noroviruses are major causes of human and animal gastroenteritis worldwide. The Norovirus $T = 3$ icosahedral capsid is made of 180 copies of the VP1 protein, as exemplified in the crystal structure of the virus-like particle (VLP) of the human Norwalk virus (NV). It was previously shown that the ca 40-nm recombinant NV VLP can be disassembled and reassembled *in vitro*. Here we report on the disassembly and self-assembly properties for the related (VP1 sequence identity of 50%) bovine Newbury2 Norovirus (NB2) VLP. Using a panel of biophysical techniques, we show that while the NB2 VLP displays disassembly properties similar to the NV VLP, NB2-VP1 shows remarkable self-assembly properties heretofore unreported for NV-VP1 or any other calicivirus capsid protein. These properties include the capabilities of self-assembling not only into regular $T = 3$ capsids but also into larger VLP (up to 76 nm in diameter) and of tolerating substitution of the spike domain for that of a distantly related Calicivirus. In conditions favoring the natural, $T = 3$ capsid, NB2-VP1 reproducibly assembles by an apparent two-phase process. Our results establish a robust new system with which to probe the dynamics of viral capsid self-assembly.

The *Caliciviridae* family of small, nonenveloped, positive-strand RNA viruses now comprises five recognized genera: Noroviruses, Sapoviruses, Lagoviruses, Vesiviruses and Neboviruses [1]. *Caliciviridae* infect humans and other animals and are serious economic and veterinary concerns worldwide. They have thus been intensely studied in the last 30 years, revealing dozens of different strains [2]. Many described *Caliciviridae* strains do not belong to any of the five recognized genera, so that several other genera have been proposed, such as Recoviruses and Valoviruses (http://www.caliciviridae.com/). Noroviruses and Sapoviruses cause gastroenteritis in humans and animals. In particular, Noroviruses are major causes of nonbacterial sporadic gastroenteritis in humans (genogroups I and II) and cattle (genogroup III). They are the leading cause of nonbacterial foodborne disease outbreaks and may soon become the leading cause of severe pediatric gastroenteritis, as the use of rotavirus vaccines becomes more widespread [3].

The most studied Norovirus is the prototype of genotype I/1, the Norwalk virus (NV[3]). Despite considerable efforts, cell culture systems are not available for Noroviruses except for a single murine Norovirus [4]. However, recombinant icosahedral $T = 3$ virus-like particles (VLP) can be produced in the baculovirus system [5]. These VLP are morphologically and antigenically indistinguishable from infectious virions isolated from patients' stool samples. The crystal structure of the NV VLP was a major breakthrough in the molecular study of Noroviruses [6]. It remains the only Norovirus crystal structure today, even though structures of two Vesiviruses (that can be produced in cell culture) have been reported since [7,8]. The VLP is made up of 180 copies of one protein, VP1. NV-VP1 can be divided in two domains (Fig. 1A). The 180 S domains make up a closed shell

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[3] *Abbreviations used:* NV, Norwalk virus; NB2, Newbury2 virus; VLP, virus-like particle; DLS, dynamic light scattering; EM, electron microscopy; SAXS, small-angle X-ray scattering; SANS, small-angle neutron scattering; I, ionic strength

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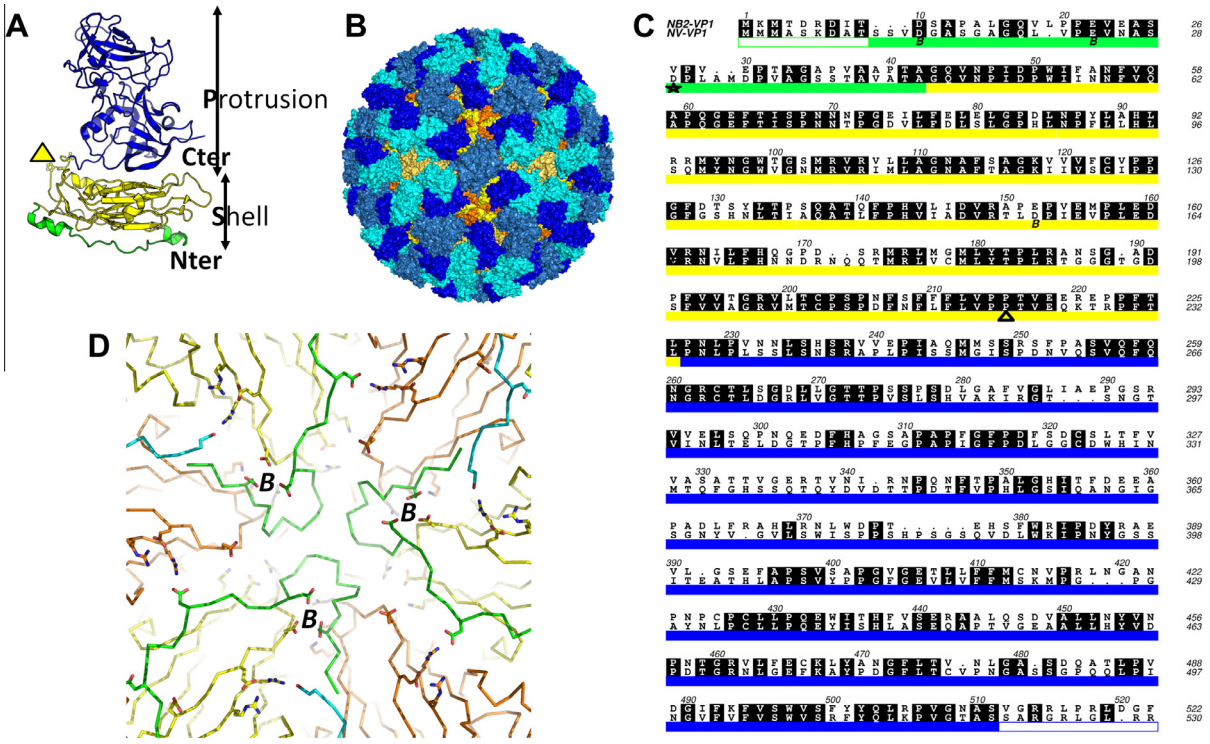

Fig. 1. Organization of the Norovirus VP1 capsid protein. VP1 is colored by domains: S (shell) domain in yellow (shell proper) and green (N-terminal arm); P (spike) domain in blue. The 'PPT' segment where the NB2 S domain connects to the NB1 P domain in the NB2_1 chimera is marked with a triangle in (A) and (C). (A) 'B' molecule abstracted from the icosahedral assembly in the crystal structure of Norwalk virus VLP (Protein Data Bank code 1IHM, [5]. (B) Surface view of the NV-VLP. The S domains and P domains of 'A', 'B' and 'C' molecules are in different shades of yellow and blue, respectively. (C) Sequence alignment of the capsid proteins of NB2 (top) and NV (bottom). The parts of VP1 that are disordered in all three 'A', 'B' and 'C' molecules of the NV VLP are blank. The start of the ordered part for molecules 'A' and 'C' is marked by a star. The three acidic amino acids brought together in the 'B' molecules around quasi 6-fold axes are marked with '***B***'. (D) View of the 3-fold icosahedral (quasi 6-fold) axis from the interior of the NV VLP. The main chain traces of the S domains of 6 subunits (3 'B' and 3 'C') are visible in this slab. The 3 'C' subunits are colored cyan (N-terminal arm) and orange. Acidic (asp and glu) and basic (lys and arg) residues are displayed as sticks. Note that all these residues are neutralized in salt bridges, except for the three clusters of three (marked with '***B***') on subunits 'B'. (For interpretation to colours in this figure, the reader is referred to the web version of this paper.)

some 30 nm in diameter, from which protrude ninety 5-nm spikes made of dimers of the C-terminal P domain (Fig. 1B). The latter thus harbors antigenic and cell tropism determinants. On the other hand, assembly determinants are harbored mainly by domain S. As in most small $T = 3$ quasiequivalent icosahedral viruses, an elaborate N-terminal arm below the body of the shell domain participates in the switch that allows the same polypeptide chain to assume three slightly different conformations, referred to as molecules 'A', 'B' and 'C' [9]. Furthermore, it has been shown that NV VLP can be disassembled and reassembled *in vitro*, the single most important parameters in these processes being pH and ionic strength [10,11]. Finally, smaller particles (presumably $T = 1$ icosahedrons) that are sometimes assembled from NV-VP1 in the baculovirus system have been found to disassemble in the same conditions as NV VLP, and to reassemble in part into the $T = 3$ NV VLP [12].

We study a close relative of NV, the prototype genotype III/2 bovine Norovirus strain Bo/Newbury2/1976/UK (NB2) [13]. NB2-VP1 (57 kDa) shares 50% sequence identity with NV-VP1 (62% between S domains, 40% between P domains, Fig. 1C). Recombinant NB2-VP1 also self-assembles *in vivo* as VLP when produced in the baculovirus system and is suitable for antigenic and receptor-binding studies [14,15]. Here we report a biophysical characterization of the VLP and their *in vitro* disassembly and self-assembly properties using electron microscopy (EM), light scattering (LS), small-angle X-ray and neutron scattering (SAXS and SANS), and low resolution X-ray crystallography. Our results reveal a very robust and versatile system that will allow precise physical characterization of capsid assembly for these most simple of mammalian viruses.

Experimental procedures

T = 3 VLP expression and purification

VLP were produced and purified as described previously [14]. Briefly, Sf9 cell monolayers were infected with recombinant baculovirus harboring the gene for NB2-VP1 (pfastbac NB2-1) at a multiplicity of infection of five and incubated in Hink's medium supplemented with 1% fetal calf serum for five days at 26 °C. VLP were extracted from freeze–thawed cells and culture medium by Freon X100 and then purified by isopycnic centrifugation in cesium chloride gradients.

We also constructed a chimeric NB2_1 capsid gene coding a VP1 protein made of the S domain of NB2 and the P domain from the Nebovirus Bo/Newbury1/1976/UK (NB1). Despite the similarity of names, NB1 is only distantly related to NB2 and is not a Norovirus [16] but a Nebovirus. A Pst1 restriction site was introduced by site-directed mutagenesis (Quickchange, Stratagene) at nucleotide position 648 of the capsid genes of the Newbury2 and Newbury1 strains of *Caliciviridae* cloned into pfastbac1 vector (Invitrogen). This position corresponds to a stretch of 3 aminoacids (PPT 214–216, NB2 numbering) in the hinge region of the two capsid proteins (Fig. 1). Fusion was obtained by ligation of the Newbury1 PstI-XhoI