Virus-like particles of calicivirus as epitope carriers

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Summary. The VP60 of rabbit haemorrhagic disease virus (RHDV), when expressed in baculovirus, self-assembles into virus-like particles (VLP) which are antigenically and immunogenically indistinguishable from native virions. When the N-terminal 30 amino acid residues of VP60 were deleted and substituted by a well characterized six residue epitope from bluetongue virus capsid protein VP7 (Btag), the fusion protein retained its ability to self-assemble into VLPs. However, the size of these particles was only 27 nm, compared to 40 nm of VLPs derived from native VP60. The antigenicity of both VP60 and the Btag was retained as evident from ELISA and Western blot analyses. When Btag was fused at the C-terminus of VP60 without deletion, the fusion proteins formed VLPs of 40 nm in size and also retained their antigenicity, but the Btag antigenicity appeared weak at this fusion site.

Introduction

Viruses belonging to the family *Caliciviridae* contain a non-segmented single-stranded, positive sense RNA genome. They are about 40 nm in size and have a characteristic morphology with cup-shaped structures on their surface [14]. Some important viruses of the family include vesicular exanthema virus of pigs, San Miguel sea lion virus, feline calicivirus, human hepatitis E virus, Norwalk virus and rabbit haemorrhagic disease virus (RHDV), also known as rabbit calicivirus (RCV) in Australia and New Zealand [9, 10, 14, 20]. Rabbit haemorrhagic disease (RHD) is an acute fatal disease of rabbits, and it is postulated that RHDV might have emerged from a pre-existing non-pathogenic rabbit calicivirus [11]. The disease was first recognized in China in 1984, and subsequently reported from different countries in Asia, Europe, Africa, and Central America [8, 12]. It is now present in Australia and New Zealand as a result of importation for biological control of wild rabbits [21]. The disease is characterized by high morbidity and high mortality in rabbits over the age of 2 months. Younger rabbits usually

survive infection. Infected rabbits die within 48 to 72 h post infection due to acute necrotising hepatitis and haemorrhages [24].

RHDV contains a genome of 7437 nucleotides (nt). The capsid protein VP60 is a cleavage product of a large polyprotein, which is encoded by ORF1 from nt 10 to nt 7042. There are reports indicating that the VP60 can also be made from a subgenomic RNA of approximately 2.4 kb [1, 15]. Considerable progress has been made on structural analysis of the capsid protein of caliciviruses. All caliciviruses possess a single capsid protein with molecular weights ranging from 59 to 65 kilodaltons (kDa). Although common among plant viruses, it is unusual for animal viruses to form capsids of a single structural protein. So far this has only been observed with caliciviruses and nodaviruses [3, 4, 6, 13]. It has been reported that 180 copies of capsid protein units are required to form a capsid in San Miguel sea lion virus [19]. The capsid protein of Norwalk virus [4] and two strains of RHDV [6, 13] have been expressed in baculovirus and shown to form virus-like particles (VLPs) which can be purified in large quantities. These VLPs are structurally and antigenically similar to native viruses. They are useful reagents for diagnosis, immunization and cell binding studies. This is of even greater importance for certain caliciviruses, like Norwalk and RHDV, since they can not be cultured in vitro. In addition, we believe that VLPs derived from single capsid protein of animal virus origin could be an ideal carrier for foreign epitopes. For this purpose, it is necessary to investigate whether capsid proteins of calicivirus, e.g., RHDV, can accommodate insertion of foreign sequences without affecting protein assembly and to locate specific regions of the VP60 as convenient insertion site(s).

In this paper we report that both N- and C-terminal of VP60 can be used as fusion sites for insertion of foreign sequences. At least 30 amino acid residues can be deleted from N-terminus of VP60 without disrupting VLP formation.

Materials and methods

Cells and viruses

Spodoptera frugiperda (clone Sf9) cells were grown at 28 °C using TC100 media (Gibco BRL) supplemented with 10% foetal calf serum plus penicillin and streptomycin. The recombinants of Autographa californica nuclear polyhedrosis virus (AcNPV) expressing RHDV VP60 proteins were propagated in suspension or monolayer cultures of Sf9 cells as described by King and Possee [5].

Recombinant plasmids and baculoviruses

The construction of bacterial expression vector pTD3 carrying six-residue epitope (Btag), pUC18-VP60F carrying full-length VP60 gene, and baculovirus transfer plasmid pVL-VP60F has been described before [13, 23]. A *Bam*HI site close to the N-terminal coding region of VP60 was used to construct an N-terminal deletion/fusion protein. Plasmid pUC18-VP60F was digested with *Bam*HI and *Eco*RI to completion to release a fragment with 90 base pair deleted at the N-terminal coding region. This *Bam*HI-*Eco*RI fragment was then cloned into pTD3 between the corresponding two sites to form pTD3-VP60′/ND11. After digesting pTD3-VP60′/ND11 with *Bgl* II and *Eco*RI, the resulting fragment was cloned into baculo-

virus transfer vector pVL1393, digested with *Bam*HI and *Eco*RI, to form pVL-VP60′/ND11. Insertion of Btag epitope at C-terminus of VP60 was carried out as follows: plasmid pVL-VP60F was digested with *Eco*RI, which is located at the C-terminal end of VP60 coding region, and treated with Mung bean nuclease and T4 DNA polymerase, followed by self-ligation and electroporation. Recombinant transformants were screened by *Eco*RI digestion of plasmid DNA and several *Eco*RI resistant clones were analysed by sequencing the C-terminal region. One such clone, designated pVL-VP60F/9, was chosen for epitope insertion. An oligonucleotide cassette containing the sequence of *Bam*HI-Btag-*Eco*RI-Stop codon-*Bgl* II was synthesized, and cloned into pVL-VP60F/9 at the unique *Bgl* II site. The resulting clone was sequenced to confirm the features introduced by the synthetic oligonucleotide cassette. The clone with correct orientation and inserted sequence was named pVL-VP60F/CD11. To construct baculovirus recombinants, insect cells (Sf9) were mock-transfected (as a negative control) or cotransfected with the linearized AcNPV DNA and plasmids pVL-VP60F (as a positive control), pVL-VP60′/ND11 and pVL-VP60F/CD11 using insectin following the manufacturer's instructions (Invitrogen Corporation, San Diego, CA, USA). The polyhedra negative recombinant plaques were picked and amplified in Sf9 cells. High titre viral stocks of three-time plaque-purified recombinants were used in subsequent expression studies.

For protein production, cells were infected with recombinants at approximately 5 to 10 multiplicity of infection and harvested 4 to 5 days post infection. The infected cells were harvested and sonicated briefly and extracted once with trichlorotrifluoroethane (Genetron). The aqueous phase was layered onto a 40% sucrose cushion and centrifuged at 35 000 rpm for 2 h using a Beckman 50.2 Ti rotor. The purified protein was resuspended in STE buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4) and the protein concentration was estimated by comparing with the known concentration of bovine serum albumin on a 10% PAGE and used for further analysis as described below. For ELISA, purified protein was diluted to 10 μg/ml, while for Westernblots 1 μg/well (for native VP60, VP60F/ND11) and 1 to 5 μg/well for VP60F/CD11.

Immunological reagents

Sheep anti-RHDV serum, and monoclonal antibodies (MAb) 2D3(3), 6D10(1), 2D4(5), 6G5(6) raised in mice against purified RHDV have been described previously [2]. The MAb 20D11 that recognises the six-residue linear epitope of bluetongue virus capsid protein VP7 was produced in this laboratory [23]. Alkaline phosphatase (AP)-conjugated and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Silenus, Melbourne, Australia. Substrates NBT/BCIP for Western blot were purchased from Promega, Madison, WI, USA.

Analysis of recombinant proteins and particle structure

Recombinant proteins were analysed by SDS-PAGE (10%) and Western blot essentially as described previously [13]. Sheep anti-RHDV antibodies were used at 1:1000 and the AP-conjugated donkey anti-sheep-IgG antibodies were used at 1:2000. Colour development was carried out using NBT/BCIP as substrates following the manufacture's protocol. Indirect ELISA was carried out as described before [13]. Aliquots of purified recombinant VP60 were examined by negative contrast and immunogold electron microscopy (NCEM and IEM, respectively). Samples for NCEM were adsorbed onto carbon coated, parlodion filmed copper grids (2 min) and stained (90 sec) with 2% phosphotungstic acid adjusted to pH 6.5 with 1 N KOH. Samples for IEM were adsorbed onto carbon coated grids as described for NCEM. Following adsorption, the preparations were blocked with 1% cold-water fish gelatine (fg, Sigma) for 10 min, incubated with RHDV-specific MAbs for 1 h at room temperature, washed
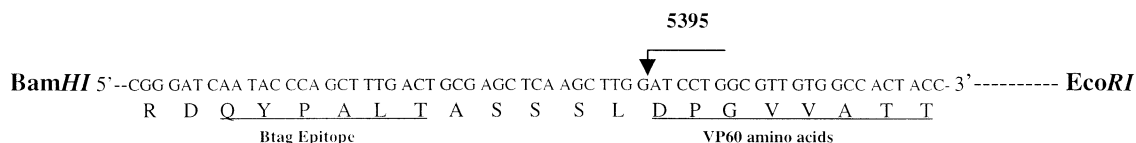
six times in phosphate buffered saline (PBS) in 1% fg (fish gelatine) and incubated with 9 nm protein A-gold for 1 h at room temperature. Preparations were then washed six times in PBS, fixed in 2.5% gluteraldehyde in PBS and stained for NCEM. All preparations were examined in an Hitachi H600 scanning transmission electron microscope at 50 kV. Haemagglutination assays (HA) were performed using human type B erythrocyte suspensions in 0.01 M PBS with 2% NaCl.

Results

Insertion of foreign epitope at the N-terminus of VP60

In our previous study we have shown that the full length VP60 protein of RHDV, when expressed by a recombinant baculovirus, forms VLPs which are morphologically and antigenically similar to native virions [13]. In the present study VP60 was modified to incorporate foreign sequences. The VP60 clone designated pVL-VP60′/ND11 contained a deletion of 30 amino acids from start codon at nt 5305 in the RHDV genome [9]. In addition, a well-characterized six-residue epitope tag (Btag) of bluetongue virus capsid protein VP7 was inserted in place of the deleted residues (Fig. 1A). Btag was chosen because this novel epitope tag has been extensively tested in bacterial, yeast and mammalian systems [23]. Two individual plaque-purified recombinants (pVL-VP60′/ND11.1 and pVL-VP60′/ND11.2) were grown in Sf9 cells and proteins produced were purified and examined by Western blot using sheep-anti-RHDV polyclonal antibodies and MAb 20D11, which specifically recognises Btag sequence (Fig. 2). A single band of about 64 kDa was observed in both blots indicating that two recombinant isolates produced an identical fusion protein as expected and that the deletion of 30 amino acids at N-terminus had no obvious effect on the antigenicity of VP60 when probed with polyclonal antibodies. In addition, the inserted Btag epitope also retained its antigenicity.

A: pVL-VP60'/ND11

5395

Bam*HI* 5'--CGG GAT CAA TAC CCA GCT TTG ACT GCG AGC TCA AGC TTG GAT CCT GGC GTT GTG GCC ACT ACC- 3'---------- Eco*RI*
         R   D   Q   Y   P   A   L   T   A   S   S   S   L   D   P   G   V   V   A   T   T
             Btag Epitope   VP60 amino acids

B: pVL-VP60F/9

6210
5'-TAT GTC GGA GCG GCC GCT GCA GAT CCC CAA TAC CCA GCT TTG ACT GAA TTC TGA AGA TCT-3'
   Y   V   G   A   A   A   A   D   P   Q   Y   P   A   L   T   E   F   -   R   S
           VP60 Amino acids   Btag Epitope   Eco*RI* stop   Bgl*II*

Fig. 1. Sequence diagram of N- and C-terminal region of VP60 after fusion with Btag epitope in plamsids pVL-VP60′/ND11 (**A**) and pVL-VP60F/CD11 (**B**). The numbers (5395 and 6210) above the nucleotide sequences of pVL-VP60′/ND11 and pVL-VP60F/9, respectively, indicate nucleotide positions of RHDV genome
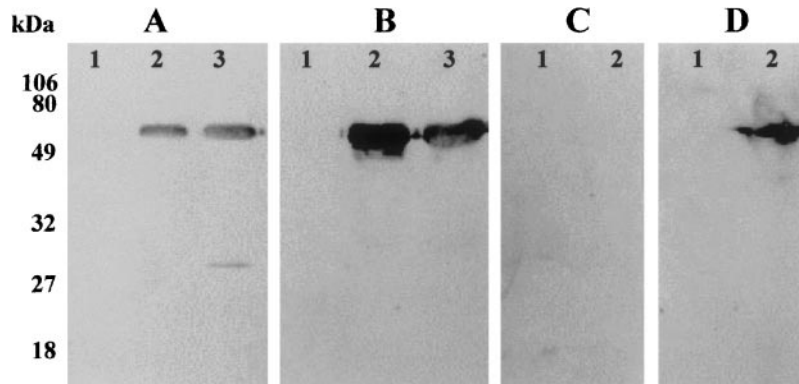
Fig. 2. Western blot analysis of purified recombinant VP60 proteins with Btag epitope. The proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed with polyclonal or monoclonal antibodies, and alkaline phosphotase-conjugated secondary antibodies except for **C**. **A** VP60 with Btag epitope replacing the 30 aa at the N-terminus, probed Btag-specific MAb 20D11. *1* Sf9 cell lysate; *2, 3* Two independent clones of VP60′/ND11. **B** Same as in **A**, except that the membrane was probed with rabbit anti-RHDV antiserum. **C** VP60 with Btag epitope inserted at the C-terminus, probed with MAb 20D11. *1* Sf9 cell lysate; *2* V60F/CD11. **D** same as **C** except that the MAb binding was detected with HRP-conjugaed anti-mouse and chemiluminescence

Reactivity of purified VP60′/ND11 protein with monoclonal antibodies was also studied in ELISA. Four RHDV-specific MAbs that recognize at least two different conformational eptitopes of VP60 protein [2] and the Btag-specific MAb 20D11 were used in the study. The results are summarized in Fig. 3. All of the MAbs reacted strongly with VP60′/ND11, and the reactivity of 20D11 was similar to RHDV-specific MAbs. These results indicated that deletion of 30 amino acids had no effect on binding of four RHDV-specific MAbs and that the Btag epitope inserted at the N-terminus could also be recognized under non-denaturing conditions.

To find out whether VP60′/ND11 proteins could assemble into VLPs, purified particles were examined by negative contrast electron microscopy. Particles about 27 nm in size were observed which had calicivirus-like morphology with cup-shaped structures on their surface similar to VLPs produced with full-length native VP60 (Fig. 4). However, immuno-gold electron microscopy studies with 20D11 showed no reactivity with the intact particles. These particles were also shown to haemagglutinate human blood type B erythrocytes similar to VLPs derived from intact VP60 (data not shown).

Insertion of foreign epitope at the C-terminus of VP60

To inverstigate if foreign epitopes can be displayed via a C-terminal fusion without disruption of VLP formation, the same Btag epitope was inserted at the C-terminus of VP60. To achieve this, plasmid pVL-VP60F was manipulated at the unique *Eco*RI site, located at the end of VP60 coding region (see Materials and
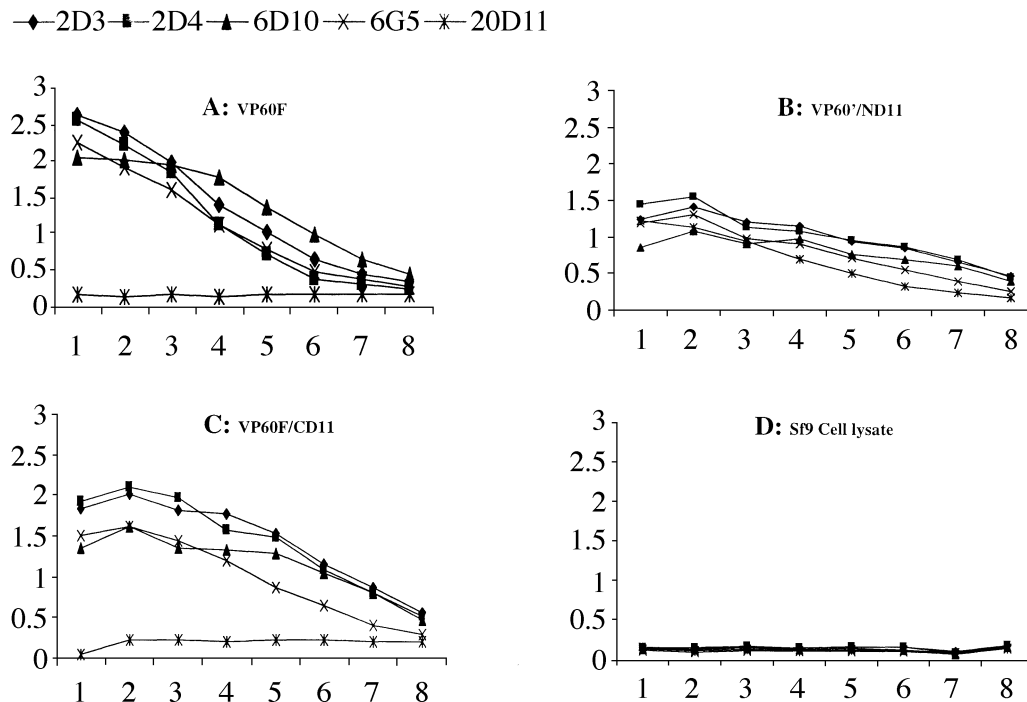
Fig. 3. ELISA results showing binding of MAbs to three different constructs of VP60 expressed in Sf9 cells. The numbers on the X-axis represent two-fold serial dilutions of MAbs supernatant (starting dilution at 1:10) while the numbers on the Y-axis represent absorbance readings at 450 nm. **A** Full length VP60 as a positive control; **B** VP60 with Btag epitope replacing the 30 aa at the N-terminus; **C** VP60 with Btag epitope inserted at the C-terminus; **D** Sf9 cell lysate as a negative control

methods for details). As a result, recombinant clone pVL-VP60F/CD11 was obtained which contained Btag epitope sequence fused to the C-terminal end without significant deletion of native VP60 amion acid residues (Fig. 1B). Recombinant protein VP60F/CD11 produced from Sf 9 cells were subject to similar analyses as for the N-terminal fusion protein. When examined by Western blot using sheep anti-RHDV serum, a major band of about 64 kDa was observed (data not shown). However, when probed with MAb 20D11 under same conditions, there was no protein band detected. The immunoblotting was repeated with increased protein concentration (5 μg/well) and chemiluminescent substrate for detection, and a band of 64 kDa was detected (Fig. 2). These results indicated that the fusion protein was expressed without significant degradation, but the antigenicity of Btag epitope was weakened when inserted at this site. ELISA further confirmed this. While four RHDV-specific MAbs reacted strongly with purified VP60F/CD11 protein, no significant reactivity was detected for 20D11 (Fig. 3).

When purified particles were examined by electron microscopy, typical calicivirus like particles with cup-shaped depressions were seen. Their size was similar to that of VLPs formed from native VP60 proteins (Fig. 4). As predicted from ELISA and Western blot analyses, MAb 20D11 did not label the VLPs
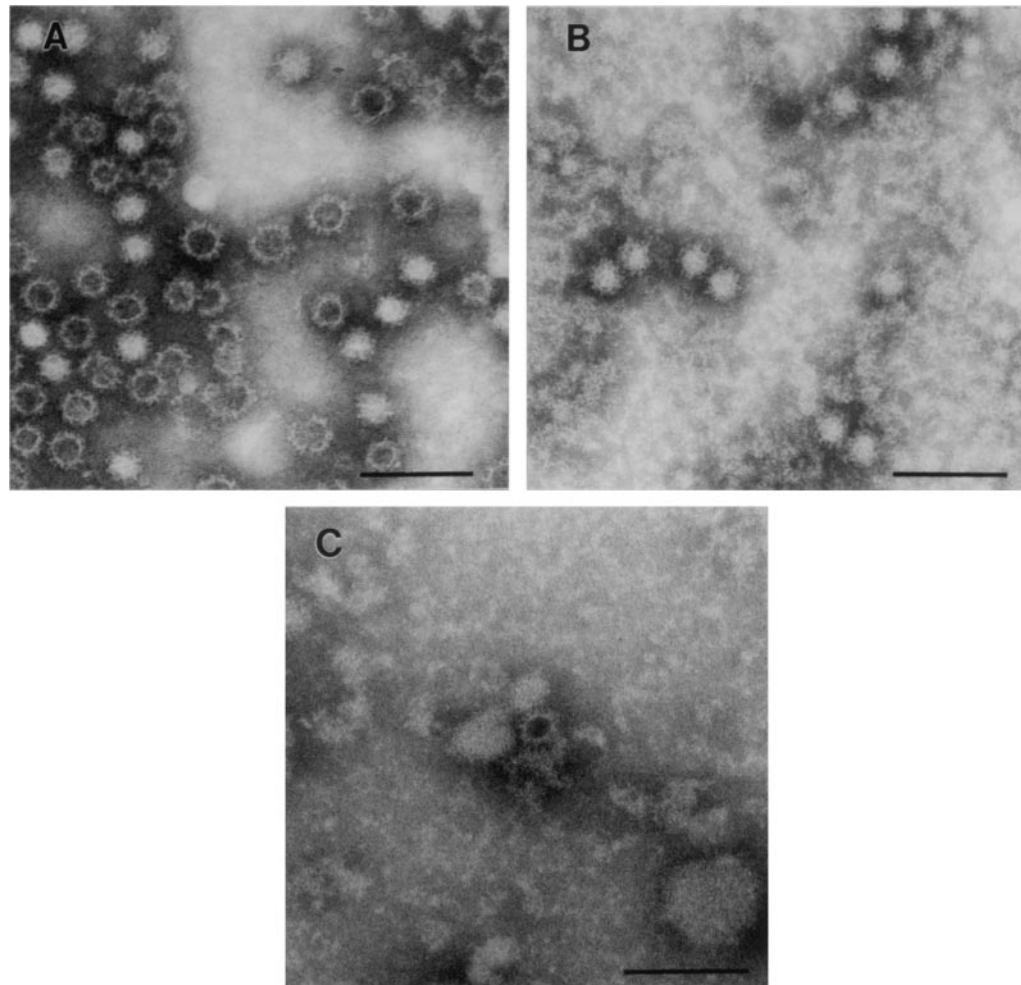
Fig. 4. Electron micrograph of virus like particles produced in Sf9 cells. **A** Full length VP60; **B** VP60 with Btag replacing the 30 aa at the N-terminus; **C** VP60 with Btag epitope inserted at the C-terminus. Bars: 100 nm

in immunogold EM (data not shown). These particles also demonstrated similar haemagglutination properties as those seen for native VLPs (data not shown).

Discussion

The VP60 protein of RHDV can accommodate foreign epitopes as shown in this study by creating fusion proteins with a well-defined six-residue epitope tag (Btag). To date, only a few animal viruses containing single capsid protein have been shown to accommodate foreign sequences at both ends of a protein.

In the N-terminal fusion, the first 30 amino acid residues were removed for the convenience of cloning. The ability of N-terminally truncated VP60 to self-assemble into VLPs suggests that truncated region of the molecule is not essential for VLP formation. It was not determined whether more residues could

be removed without affecting VLP formation. Recently, Li et al. [7] reported self-assembly of VLPs from capsid protein of hepatitis E virus (HEV), another calicivirus. It is interesting to note that HEV VLPs could only be produced from truncated capsid proteins expressed from Tn5 insect cells. The protein involved in VLP assembly lacked the first 111 aa at N-terminus, and also approximately 50 aa from the C-terminus. The results from our studies are consistent with those of Li et al. [7] indicating that neither the N-terminus nor the C-terminus is involved in protein-protein interaction during VLP assembly. Although it is too early to conclude, we speculate that this general feature could apply to all calicivirus capsid proteins due to their known similarties of primary sequences as well as capsid structures.

One unresolved issue in calicivirus VLP assembly is the relationship between the size of the capsid protein and the size of the assembled VLPs. In our study, replacement of 30 aa at the N-terminus with the Btag produced VLPs of 27 nm. On the other hand, addition of Btag at the C-terminus produced VLPs of 40 nm, which are similar or identical in size to those produced from wild type VP60. The size reduction from 40 nm to 27 nm in the N-terminal fusion construct is unlikely to be solely due to the decrease in length of fusion proteins since the net difference between the VP60′/ND11 and wild type VP60 proteins is only 17 aa. Rather we hypothesize that 27-nm VLPs were produced from less assembling units than that of 40-nm VLPs. This notion is supported by a recent publication by White et al. [22], in which the authors demonstrated two types of Norwalk virus VLPs (23-nm and 38-nm particles) produced from same 58-kDa protein in Sf9 insect cells, i.e., an identical assembly unit produced two different sizes of VLPs depending on how VLPs are assembled. They hypothesized that the 38-nm VLPs were assembled from 180 units with a T=3 symmetry whereas the 23-nm VLPs were assembled from 60 units with a T=1 symmetry. There are striking similarities between these two calicivirus capsid proteins in their ability to produce two different sizes of VLPs. In both cases, size of the smaller VLPs is about 60–70% of the larger ones.

The hypothesis that VLP size change is a result of fewer assembly units rather than gross change in the protein unit was further supported from our studies on antigenicity and cell binding properties. Both types of VLPs reacted not only with different polyclonal antibodies in ELISA and Western blot, but also with four monoclonal antibodies which were known to recognize conformational epitopes [2, 13]. In addition, the 27-nm VLPs also retained their ability to haemagglutinate human type B erythrocytes. Collectively, these results suggest that the overall conformation of VP60 molecules has not changed as a result of N- or C-terminal deletion/fusion.

The antigenicity of the fused Btag was examined by ELISA, Western blot and immuno electron microscopy for both types of VLPs. When fused at the N-terminus, the Btag was detectable by MAb 20D11 in both ELISA and Western blot but not in immuno electron microscopy. The Btag antigenicity was much weaker when fused at the C-terminus, and could only be detected in Western blot using highly sensitive chemiluminiscent substrate when the protein

concentration was increased to about 5 µg/well. This "epitope masking" effect is not uncommon, and is believed to be contributed from a combination of epitope location in relation to protein domain structure and the nature of flanking amino acid residues. Although it is not possible to predict whether fusion of Btag with a particular sequence will enhance or weaken epitope antigenicity, it has been observed that presence of a glutamic acid residue (E) immediately following Btag sequence could weaken eptitope reactivity [23]. It was interesting to note that an E residue was present at this position in the VP60F/CD11 fusion construct. The failure of immuno gold labeling of 27-nm VLPs with MAb 20D11 could be explained by the inaccessibility of epitope due to its internal location. The 3-D structure of Norwalk virus VLPs assembled in baculovirus expression system has been determined by Prasad et al. [17], which indicates that the C-terminus of the capsid protein is exposed on the VLP surface while N-terminus is located inside the empty particle. Unless RHDV VP60 is radically different, which is most unlikely, we believe that the lack of reactivity with 20D11 in immuno EM is most likely due to the fact that Btag fused at N-terminus was buried inside VLPs. This may be advantageous for certain applications since the buried epitopes are better protected from proteolytic attack during protein synthesis and purification.

We have previously shown that RHDV VLPs are very efficient in inducing protective immune response when inoculated into rabbits by intramuscular route [13]. Recently, it has been reported that these VLPs can be administered orally to rabbits in very small doses (0.5 µg or 3 µg/rabbit) and obtain solid protection against a lethal challenge [16]. Their data suggest that VLPs are comparable to immunoenhancing proteins such as cholera toxin in stimulating the mucosal immunity. These properties and the ability of VLPs to accommodate epitopes at either end of the molecule, as we have shown in this study, may lead to novel platforms for vaccine development and delivery.

In summary, our results demonstrate that VLPs derived from RHDV VP60 could be used as a carrier for foreign epitopes. The fusion of foreign sequences could be achieved at either N- or C-terminus of the protein. Since only a single protein is involved in VLP formation, this epitope carrier system will be easier to use than some of the multi-subunit system, such as the two-component bluetongue virus capsid core like particles (CLPs) [18].

Acknowledgements

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