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Vaccine development through terminal deletions of an infectious bursal disease virus protein 2 precursor variant

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ABSTRACT

VP2 is the primary host-protective immunogen of infectious bursal disease virus (IBDV), the agent that causes the highly contiguous infectious bursal disease (IBD). Previous studies have shown that a C-terminal his-tagged 452 amino acid residue VP2 precursor variant (VP2-452H) can form an immunogenic subviral particle (SVP). A set of his-tagged N- and C-terminal VP2-452 deleting mutants (designated as N5-452H, N10-452H, N20-452H, N40-452H, VP2-441H, VP2-437H, VP2-411H and VP2-399H) was expressed in insect cells to discover the role of both N- and C-termini on the assembly of SVP and to develop an efficient SVP-based vaccine. Among these mutants, the expression level of N5-452H was the highest. Results of ultracentrifugation and electron microscopy also indicated that mutants of N-terminal deletion N10-452H, N20-452H and N40-452H or C-terminal deletion VP2-411H and VP2-399H lost the capability to self-assemble SVP. The other mutants, N5-452H, VP2-441H and VP2-437H, formed SVP. Additionally, SVP formed by N5-452H could not only be single-step purified by immobilized metal-ion affinity chromatography (IMAC), but it could also induce a high titer of neutralizing activity to protect chicks from the infection of IBDV at a low dosage (0.2 μg), suggesting that SVP formed by N5-452H can be an alternative vaccine candidate for the prevention of IBD.

1. Introduction

Infectious bursal disease virus (IBDV), a member of the genus *Avibirnavirus* in the *Birnaviridae* family, causes a highly contagious disease in young chickens. The infection results in a functional loss of the bursa of Fabricius and a severe immuno-depression, making chickens prone to other diseases and rendering vaccination ineffective against infectious bronchitis, Marek's disease, and Newcastle disease [1]. In general, IBDV infection is controlled by vaccinating with an attenuated strain of IBDV or by transferring high levels of maternal antibodies induced by the administration of live and non-live IBDV vaccines to breeder hens [2]. Infectious bursal disease continues to pose an important threat to the commercial poultry industry, especially after the emergence of the very virulent infectious bursal disease virus (vvIBDV), because conventional vaccines used in the field are gradually losing their effectiveness [3].

The IBDV genome contains two segments of double-stranded RNA, designated as A and B, respectively [4]. Segment A encodes a 108-kDa polypeptide that is self-cleaved to produce precursor VP2 (pVP2), VP3, and VP4. In mature virions, pVP2 with 512 amino acid residues are processed into VP2 by cleaving the C-terminal 71 amino acid residues [5]. VP2 protein is a single outer protein and the primary host-protective immunogen of IBDV [6,7], and it contains the antigenic regions responsible for the elicitation of the neutralizing antibodies [7–9]. VP2 has been expressed by prokaryotic systems [10], yeast systems [11], a recombinant fowlpox virus [12], an inducible vaccinia virus recombinant [13], as well as baculovirus recombinants [14]. Among these, a vaccine based on VP2 expression in a *Pichia pastoris* is commercially-available after a large-scale vaccination test [11]. This approach demonstrated that a recombinant protein subunit vaccine is an alternative means of combating and controlling this poultry disease. The VP2 gene was expressed in *Escherichia coli* in our previous study, but the expression level was too low to commercialize the product, at this point, as a subunit vaccine

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against IBDV [10]. The VP2-452H monomer protein can self-assemble into a highly immunogenic subviral particle (SVP) with about a 25 nm diameter in insect cells [14,15]. VP2-452H is fused with a his-tag at the C-terminus of VP2-452, a 452 amino acid residue of pVP2 variant, to simplify the purification by immobilized metal-ion affinity chromatography (IMAC), and this did not alter its capability to form an SVP capsid and the immunogenicity it induced [15]. In the present work, we have further explored the terminal regions of VP2-452 protein responsible for a high expression level of SVP in insect cells with a view to develop a more efficient vaccine.

Since the neutralizing domains are highly conformational, successful vaccine development requires systems where the recombinant products mimic the authentic proteins and their conformation. In this context, capsid-like particles have been powerful tools in a number of viral diseases since they resemble the native viral capsids structurally and immunologically [16]. Available high resolution crystallographic studies indicate that the VP2 protein inside the icosahedral SVP with a T number of 1 ($T = 1$) folded mainly into a shell (S) domain and a protrusion (P) domain, both with a Swiss-roll topology, plus a base (B) domain [17]. The surface loops of the P domain of VP2 SVP are responsible for the interaction with the receptor [18], the recognition by antibodies [9] and the binding with the immobilized Ni^{2+} ions as recently demonstrated [19]. The B domain consists of N- and C-terminal α-helices that contour the capsid's inner surface. However, the first 10 and the last 22 amino acid residues of VP2-452 as well as an extra his-tag, located in domain B, were not observed according to our X-ray structural model at 2.6 Å, probably because they are highly dynamic [17]. Consequently, their function on the capsid assembly is unknown. As seen from other viral coat proteins, a number of amino acids at the termini of a polypeptide chain usually lie outside the actual barrel structure [20]. Thus, a minor deletion at N- or C-termini of the coat protein may not affect the formation of the capsid and the resulting immunogenicity [21–26]. In this study, a deletion of amino acid residues at the N- or C-terminal region of VP2-452 was carried out to study the effect on this $T = 1$ capsid formation. Consequentially, the region responsible for the formation of the capsid was identified and the capsids formed by the deletion mutants were tested for IMAC purification efficiency and for immunogenic property evaluation for the development of an alternative IBD vaccine.

2. Materials and methods

2.1. Viruses, cells and animals

A local isolate strain of IBDV, P3009, and a very virulent strain 93/4/6 of IBDV (vvIBDV 93/4/6) were prepared as previously described [15]. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and recombinant baculovirus stocks were propagated in *Spodoptera frugiperda* (*Sf*9) cells (American Type Culture Collection 1171) using TNM-FH medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Bet Haemek, Israel) in tissue culture flasks (Corning, NY) at 28 °C [27]. High-five™ (Hi-5) cells (purchased from Invitrogen) were routinely cultured and passaged in ESF-921 medium (Expression System LLC, Woodland, CA). Total insect cell counts and viability were determined as described previously [28]. Specific pathogen free (SPF) chickens were purchased from Branch Institute of Animal Drugs Inspection, Animal Health Research Institute, Council of Agriculture, Executive Yuan (Miaoli, Taiwan).

2.2. Cloning of IBDV truncated VP2 genes into a baculovirus transfer vector

Polymerase chain reaction (PCR) was performed, using plasmid pBluescript-VP2 as a template [29], to generate the N-terminal and C-terminal truncated VP2 gene fragments. The sequences of four forward primers used are (5′–3′) P4F: CGATCGCTAGCGATGACAAAC; N15F: ACGCTAGCATGGATCAAACCCAACAGATT; N30F: ACGCTAGCATGATTGTTCCGTTCATACGG; N57F: GAGAATTCTGATGCCAACAACCGGAC; N120F: ACGCTAGCATGGAGACCTCGACCTACAATTTG, respectively, and the sequences of four reverse primers are (5′–3′) R1323H: CGGAATTCCT*AGTGATGGTGATGGTGATGGT*GCTCCTGCAATCTTCAG; R1311H: TTGAATTCT*AATGGTGATGGTGATGGTGC*TTCAGGGGAGAGTTGAG; R1233H: CGGAATTCCT*AGTGATGGTGATGGTGATGC*TTGATGCCAAGACGTTC; R1197H: CGGAATTCT*AATGGTGATGGTGATGGTG*TTTGTGTAGTTCATGGCT; VP2B: GCGAATTCT*AATGGTGATGGTGATGGTG*CCTTATGGCCCGGATTAT, respectively, where the restriction enzyme sites are underlined. All reverse primers are inserted 18 nts for the fusion of a His-tag of six histidine residues (italic) at the C-terminus of the truncated proteins to assist protein purification using IMAC. Primer pairs: (N15F, VP2B); (N30F, VP2B); (N57F, VP2B); (N120F, VP2B); (P4F, R1323H); (P4F, R1311H); (P4F, R1233H); (P4F, R1197H) were used to generate six gene fragments of VP2 mutants, N5-452H, N10-452H, N20-452H, N40-452H, 441H, 437H, 411H and 399H, respectively. The purified PCR products were digested, purified, and ligated to pBlueBac4 transfer vector (Invitrogen, San Diego, CA) as previously described [15]. The resulting recombinant plasmid was transformed into competent *E. coli* JM109 for selection, isolated and analyzed by restriction enzyme digestion and sequenced. Thus, four N-terminally truncated constructs of VP2, i.e., pBB4N5-452H, pBB4N10-452H, pBB4N20-452H and pBB4N40-452H were prepared. Similarly, PCR products of the C-terminally truncated VP2 were cloned into pBlueBac4 to form plasmids pBB4VP2-441H, pBB4VP2-437H, pBB4VP2-411H and pBB4VP2-399H.

2.3. Generation of recombinant baculoviruses expressing VP2-452-truncated fusion proteins

The generation of recombinant baculovirus expressing VP2-452H, also named as rVP2H, protein was described previously [15]. Following the verification of all recombinant transfer plasmids by DNA sequencing, recombinant baculoviruses were obtained by cotransfecting plasmids pBB4N5-452H, pBB4N10-452H, pBB4N20-452H, pBB4N40-452H, pBB4VP2-441H, pBB4VP2-437H, pBB4VP2-411H and pBB4VP2-399H, respectively with linear AcMNPV DNA into *Sf*9 cells. Then, putative recombinant baculoviruses were isolated as previously described [15] and amplified in T-25 cm^2 flasks. The plaque-pure recombinant baculoviruses containing sequentially truncated VP2 genes with His-tag, vN5-452H, vN10-452H, vN20-452H, vN40-452H, v441H, v437H, v411H, and v399H, were confirmed by PCR analysis following the procedure previously described [30].

2.4. Expression and production of SVP formed by mutant proteins

*Sf*9 cells, cultured in shaking flasks at 28 °C, were infected with different recombinant baculoviruses for the expression of mutant proteins. The infected cells were harvested 3 days post-infection and then subjected to centrifugation. The expression level of mutant protein in the pellet was examined by Western blotting analysis, and the supernatant was used as a viral stock to infect Hi-5 cells for the production of proteins. An end-point dilution method was chosen to determine the titer of a recombinant virus in the viral stock according to the method previously described [27]. For the production of various mutants to be purified for morphology observation, Hi-5 cells were cultured using an ESF921 serum-free medium and infected with the recombinant baculoviruses at a multiplicity of infection of 10 when the viable cell density was near 2×10^6 cells/ml in 500 ml spinner flasks [28]. After infection, cells were harvested when the viability fell below 70%, and the expression level of each recombinant protein was estimated by Western blotting analysis.

2.5. Purification of SVP formed by mutant proteins using ultracentrifugation

The harvested Hi-5 cells were centrifuged at 5000 × *g* for 20 min. The resulting cell pellets were resuspended in 3 ml of TNE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.6) and, then, sonicated on ice three times for 10 s with a 30% pulsed duty cycle (Vibra cell, Sonics & Materials, Inc., Newtown, CT). Insoluble cell debris was removed by centrifugation at 10,000 × *g* for 20 min and the supernatant was loaded on a 25% (w/v) sucrose solution and subjected to centrifuging at 35,000 rpm and 4 °C for 3 h in an SW41 Ti rotor. After sucrose cushion, the pellet was resuspended in 0.5 ml of TNE buffer, and then subjected to 20–40% cesium chloride (CsCl) gradient centrifugation. The solution in the centrifuge tube was collected at 0.5 ml/fraction and each fraction, verified by buoyant density measurement, was analyzed using either Western blot or electron microscopy (EM) for morphology observation.

2.6. Purification of SVP formed by mutant proteins using immobilized metal-ion affinity chromatography

The cell pellet from a 500 ml spinner flask of Hi-5 cell culture was resuspended in binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.8) and sonicated as mentioned above. Insoluble cell debris was removed by centrifugation at 3000 × *g* for 15 min. Ni-NTA resin (QIAGEN, Valencia, CA) was then added into the cell lysate solution for 2 h, and then the virus particle-bound resin was loaded onto a column (2.5 × 10 cm, Bio-Rad) and the unbound materials were collected as flow-through. To remove more impurities from the resin, the column was washed by 10× resin volume of binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.8) and then followed by 10× resin volume of washing buffer (20 mM NaH_2PO_4, 0.5 M NaCl, pH 6.3). The desired mutant protein was then eluted by elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, pH 4.0). Fractions were monitored at OD_{280} by a U-2001 spectrophotometer (Hitachi Ltd., Tokyo, Japan), with the washing buffer as a blank. The eluate from the putative peak containing the desired protein was collected, concentrated and analyzed by