ORIGINAL ARTICLE

The VP5 protein of infectious bursal disease virus promotes virion release from infected cells and is not involved in cell death

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Abstract The VP5 protein of infectious bursal disease virus (IBDV) was shown in previous reports to be involved in the cytopathogenicity of IBDV. Here, using a rescued VP5-deficient IBDV infectious clone, it was demonstrated that a lack of VP5 expression significantly hinders the release of viral progeny from infected cells but does not block intracellular virus production. Monoclonal VP5-expressing Vero cells did not exhibit induction of cell death. Using VP5-specific mAbs generated in our laboratory as a tool, it was shown by flow cytometry analysis that VP5 was detectable on the surface of IBDV-infected and monoclonal VP5-expressing Vero cells and bursal cells in IBDV-infected chickens. Taken together, these data suggest that the VP5 protein is involved in regulation of the release of intracellular IBDV virions and may be used as a cell-surface marker for detecting IBDV-infected cells in FCM analysis. This study contributes to the further characterization of the VP5 protein, which will allow a better understanding of the mechanism of IBDV pathogenicity.

Introduction

Infectious bursal disease virus (IBDV), a member of the genus *Avibirnavirus* within the family *Birnaviridae*, is the causative agent of infectious bursal disease (IBD). IBDV causes severe immunodeficiency in young chickens by

destroying immature antibody-producing B lymphocytes in the bursa of Fabricius (BF), which is followed by bursal atrophy. This renders the animal increasingly susceptible to disease and decreases the effectiveness of vaccination against infectious diseases [9, 22]. Two serotypes of IBDV (serotype 1 and 2) can be differentiated by a neutralization test [7, 12]. All pathogenic IBDVs are serotype 1 strains; serotype 2 strains neither cause disease nor protect against infection by serotype 1 [5, 6].

IBDV particles have a non-enveloped, icosahedral capsid with a diameter of about 60 nm and contain two segments of double-stranded RNA of 3.3 kb (segment A) and 2.9 kb (segment B) [3, 13]. Segment A contains two partially overlapping open reading frames (ORFs). The larger ORF encodes the structural proteins VP2 and VP3 and a protease, VP4 [1, 4, 14]. The smaller ORF encodes the highly conserved, basic and cysteine-rich class II membrane protein VP5 [11, 16]. While the VP5 protein is not present in the virion [2] and is not required for viral replication [16], it is thought to play an important role in cytopathogenicity [25]. Its accumulation in the host cell membrane is thought to be involved in the release of viral particles and in the induction of apoptosis [11, 26]. Indeed, research using the yeast two-hybrid system has indicated that the central domain of VP5 mediates interaction with itself [20], supporting the hypothesis that VP5 molecules coassemble at the host plasma membrane to form a pore structure [19, 20]. Hence, it would be interesting to evaluate whether mutations of conserved amino acids in the putative transmembrane region of VP5 would affect its membrane-permeabilizing activity [20]. However, in elegant experiments using a chimeric IBDV virion, Schröder et al. [18] recently showed that VP5 is not responsible for the differential pathogenicity of serotypes I and II of IBDV. Consequently, we are interested in exploring the role of

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VP5 to better understand the factors underlying the pathogenicity of IBDV. In this study, monoclonal cell lines expressing the VP5 protein of IBDV were constructed, and the function of VP5 protein inhibiting IBDV release from infected cells was confirmed.

Materials and methods

Virus strain, cell lines, antibody and chickens

The NB strain of IBDV, a pathogenic serotype I virus isolated in China, was adapted to growth in chicken embryo fibroblasts (CEF) [27, 32]. Vero cells permissive for IBDV infection were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and were maintained in MEM (Gibco BRL, USA) supplemented with 10% newborn calf serum (NCS). Specific-pathogen-free (SPF) Leghorn chickens and embryonated chicken eggs were purchased from Beijing Merial Vital Laboratory Animal Technology Co. Ltd, Beijing, China. Primary CEF cells were prepared from 10-day-old embryonated SPF chicken eggs and maintained in MEM supplemented with 10% NCS. Both cell lines were used for propagation of IBDV. The monoclonal antibody (mAb) to VP4 protein of infectious bursal disease virus was generated in our laboratory [23].

Preparation of monoclonal antibody (mAb) against VP5 protein

To generate mAbs to VP5, recombinant VP5 protein expressed in *E. coli* was prepared as an immunogen for mouse vaccination. Briefly, the vector pCI-A containing the complete genomic A segment of IBDV (unpublished data) was used as a template for PCR. A 439-bp cDNA fragment encoding the complete VP5 protein was amplified by RT-PCR with primers VP5-up (Table 1) containing an EcoRI site and VP5-down (Table 1) containing a SalI site, which were designed according to the cDNA sequence of segment A of IBDV strain NB (GenBank accession no. AY319786). RT-PCR was performed at 95°C for 5 min, 95°C for 1 min, 58°C for 1 min and 72°C for 2 min for 30 cycles, with a final elongation step for 5 min at 72°C. Then, PCR products were inserted into the prokaryotic expression vector pET-28a (+) (Novagen, Madison, WI, USA). The resulting plasmid, pET-VP5, was confirmed by PCR, and its DNA sequence was verified. It was then used to transform BL21 (DE3) E. coli for expression. His-tagged VP5 was optimally expressed in E. coli as insoluble inclusions, induced by a final concentration of 1 mM isopropyl- β -D-thiogalactoside (IPTG). The expressed protein became soluble after treatment of E. coli cells with lysis buffer containing urea, NaH₂PO₄ and Tris. SDS-PAGE analysis showed that the His-VP5 fusion protein appeared to have a molecular weight of approximately 23 kDa (data not shown). After purification with an Ni-NTA affinity chromatography column (Novagen, Madison, WI, USA), His-VP5 protein could be recognized by anti-His mAb in western blot analysis (data not shown).

Five SPF BALB/c mice were immunized subcutaneously (S/C) with rVP5 (50 μ g rVP5 per mouse) emulsified in complete Freund's adjuvant (CFA, Sigma–Aldrich, St. Louis, MO) and boosted twice at 2-week intervals with rVP5 emulsified in incomplete Freund's adjuvant (Sigma) after the first immunization. Two weeks after the third immunization, the vaccinated mice were injected intraperitoneally with 0.1 mg of rVP5. Three days after the final immunization, spleen cells were fused with SP2/0 cells as described by Kohler and Milstein [10]. The resulting hybridoma cells were cultured in RPMI-1640 containing hypoxanthine-aminopterin-thymidine (HAT) and hypoxanthine thymidine (HT) for 10–14 days. Further selection and confirmation of mAb recognition of VP5 protein were

Table 1 Oligonucleotide primers used in PCR

Name	Sequence	Orientation	Position (nt)
VP5-up	CCGAATTCATGGTCAGTAGAGATCAGACAAACG	+	96–121
VP5-down	GATCGTCGACTCACTCAGGCTTCCTTGGAAGGTC	_	511-531
AT7	TAACTAGTTAATACGACTCACTATAGGGGATACGAT	+	1–9
A5 m	CTATAGGGGATACGATCGGTCTGA	+	1–17
VP5F	GCTATCATTGATTGTCAGTCGA	+	97-108
VP5R	CTGACAATCAATGATAGCGTT	_	84-104
A3 m	CCACGCGTGGGGACCCGCGAACGGAT	_	3,242-3,259
BT7	TA GAGCTC TAATACGACTCACTATAGGGGATACGAT	+	1–9
B3 m	CCACGCGTGGGGGGCCCCCGCAGGCGAA	_	2,809-2,827

Nucleotide positions correspond to the published sequence of IBDV strain NB p38 [32]. T7-promoter-specific sequences are underlined, introduced restriction sites are indicated in bold and mutated nucleotides are shown in italics. The orientation of the virus-specific primer sequence is shown as sense (+) or antisense (-)

carried out by indirect enzyme-linked immunosorbent assay (ELISA), using cell lysates of *E. coli* BL21 (DE3) and rVP5 as antigens. Hybridomas that secreted rVP5 antibodies were subcloned three times by limiting dilution. The reaction of mAbs to native VP5 was assayed using VP5 protein expressed in IBDV-infected CEF and Vero cell monolayers as antigen as described previously [28]. Briefly, CEF and Vero cells inoculated with IBDV were cultured for 36 h. The cells were washed twice with PBS and fixed with 4% paraformaldehyde. IFA assays were performed as described below. In addition, the mAbs against rVP5 were identified by western blot assays using rVP5 as antigens. The IgG subtype of each mAb was determined using SBA Clonotyping System/HRP (Southern Biotechnology, Birmingham, AL, USA).

Construction of the eukaryotic expression vector, transfection and cloning

To subclone the VP5 gene into the eukaryotic expression vector pCI-neo (Promega, USA), a 439 bp-cDNA segment encoding the VP5 protein was obtained as described above. The resulting plasmid, designated pCI-neo-VP5 (Fig. 1a), was used to transform Top10 competent E. coli cells (Invitrogen, USA), and cloned fragments were identified by PCR and sequencing. Finally, the plasmids were isolated using a high-purity plasmid purification system (Marligen, USA) and linearized with BamHI. Vero cells were grown to 95% confluence and transfected with linearized recombinant plasmid using LipofectamineTM (Invitrogen) according to the manufacturer's instructions. Forty-eight hours post-transfection (h.p.t.), Vero cells transfected with the pCI-neo-VP5 were trypsinized and persistently selected in MEM containing neomycin G418 (500 µg/mL; B. M. Scientific) for 2-3 weeks, until G418-resistant "cell islands" formed. Every G418-resistant cell island was cloned and selected three times by limiting dilution in MEM containing G418 to obtain a single cell clone. The G418-resistant cell clones that grew well were selected for further analysis. Non-transfected cells and cells that were mock-transfected with pCI-neo vectors were analyzed in parallel as controls.

PCR and Southern blot assays of genomic DNA

To detect chromosomal integration of the VP5 gene in the cloned, pCI-neo-VP5-transfected Vero cells, genomic DNA was isolated from each G418-resistant clone by spooling, as described previously [17]. Genomic DNA was treated with RNase and subjected to PCR as described above. Southern blot analysis was performed using an alpha-³²P-labeled VP5 probe (300 bp) prepared with a random prime DNA labeling kit Ver.2 (TaKaRa, Japan) as described previously [17]. Genomic DNA from Vero cells



Fig. 1 Schematic representation of the vectors constructed in this study. **a** Construction of the pCI-neo-VP5 vector. The VP5 gene sequence with a TGA termination codon was cloned into a pCI-neo vector digested with *Eco*RI and *Sal*I.**b** Construction of the pCI-neo-A-VP5⁻ vector. The IBDV-A gene sequence lacking the VP5 gene initiation codon (ATG) was cloned into the *Spe*I and *Mlu*I sites of the pCI-neo vector. **c** Construction of the pCI-neo-B vector. The IBDV-B gene sequence was cloned into a pCI-neo vector digested with *Sac*I and *Mlu*I. H_{CMV}: human cytomegalovirus; MCS: multiple cloning sites

transfected with the parental pCI-neo vector was used as a negative control.

RT-PCR and northern blot analysis of mRNA transcription

Transcription analysis was carried out for each G418-resistant pCI-neo-VP5-transfected clone that gave a positive Southern blot result. Total cellular RNA was

prepared with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Analysis of VP5 transcription was performed using the One Step RT-PCR Selective PCR Kit Ver.1.1 (TaKaRa), and northern blot analysis with an alpha-³²P-labeled VP5 probe, as described previously [17].

Indirect immunofluorescence assay

Indirect immunofluorescence assays (IFAs) were performed as described in ref. [28]. Briefly, a plate of cells was fixed with 4% paraformaldehyde (in PBS, pH 7.4) for 30 min at room temperature and allowed to air dry. The fixed cells were blocked with PBS (717 mmol/L K₂HPO₄, 283 mmol/L KH₂PO₄, pH 7.2) containing 5% dry skimmed milk for 60 min at 37°C. The plate was washed three times with PBST (PBS with 0.25% Tween-20). Mouse anti-VP5 mAb or mouse anti-VP4 mAb (1:100, diluted with PBS containing 5% dry skim milk) was added to the plate, which was incubated for 60 min at 37°C. After three washes in PBST, diluted FITC-conjugated goat anti-mouse IgG (KPL, USA) was added to the wells, and the plate was incubated for 60 min, followed by three PBST washes. Additional nuclear staining with 4',6-diamidino-2-phenylindole (Sigma) was performed as described previously [28]. The cells were then observed under an inverted fluorescence research microscope (Olympus, Japan) for IFA analysis. IBDV-infected cells were used as positive control.

Construction of a VP5-deleted infectious IBDV clone

Vectors pCI-A and pCI-B, containing the complete genomic A and B segments, respectively, of IBDV strain NB (unpublished data) were used as templates. (The GenBank accession number of the cDNA sequence of segment B is AY654284.) To construct a mutant cDNA clone of segment A lacking the initiation codon of the VP5 gene, using a previously reported method [15], we amplified two DNA segments with unique primer pairs (A5 m plus VP5R and VP5F plus A3 m; Table 1) and used the resultant segments as a template for fusion PCR with AT7 and A3m primers. After replacing the VP5 gene initiation codon (ATG) of the A segment with "ATT", an amplified fragment flanked by *SpeI* and *MluI* sites was cloned into pCI-neo to yield the



Fig. 2 Reactivity of VP5-specific mAbs with natural VP5 protein in IBDV-infected CEF and Vero cells by IFA. IBDV-infected CEF **a** and Vero cells **b** were detected using mAbs 1B2, 1B9, 2E6, 2E8, 3A4, 4B1,

4B7 and 5C2, specific for VP5. **a** and **b** show that the anti-VP5 mAbs binds transiently to naturally expressed VP5 protein in IBDV-infected CEF and Vero cells. *Bar* 20 μ m (original magnification ×40)



Fig. 3 Immunoactivity of mAbs to rVP5 protein by western blot. Purified rVP5 separated by SDS-PAGE was used as antigen for western blot analysis with mAbs 1B2, 1B9, 2E6, 2E8, 3A4, 4B1, 4B7 and 5C2, specific for rVP5 protein. Lysates of *E. coli* BL21 (DE3)

plasmid pCI-neo-A-VP5⁻ (Fig. 1b). We also amplified the whole segment B, flanked by *SacI* and *MluI* (BT7 and B3 m; Table 1), and cloned the resultant segment into pCI-neo to obtain plasmid pCI-neo-B (Fig. 1c). Plasmids pCI-neo-A-VP5⁻ and pCI-neo-B were digested with *MluI*. After purification with TE phenol (pH 8.0), in vitro transcription was performed using the linearized plasmids as template, according the protocol of the T7 RiboMAXTM Express RNAi System (Promega). Vero cells were co-transfected with transcripts of mutant segment A and of segment B, using LipofectamineTM 2000 (Invitrogen). The resulting progeny were designated recombinant VP5⁻/IBDV.

The role of VP5 protein in replication and cytopathogenicity of IBDV

To determine the role of VP5 protein in IBDV infection, a CEF monolayer was inoculated with the parental IBDV or VP5⁻/IBDV at 500 TCID₅₀. The cell supernatant, cellular monolayer (CM), and cell supernatant plus cellular monolayer (CSC) were collected at various time points post-infection (p.i.) for virus titration. All virus samples were freeze-thawed three times, followed by centrifugation at 2,000×*g* for 10 min. Finally, the virus titer was determined by end-point dilution (tenfold serial dilution) on CEF monolayers cultured in 96-well plates. The cytopathogenic effect (CPE) was observed every day until 7 days p.i., and the 50%-tissue culture infectious dose (TCID₅₀ ml⁻¹) was determined according to Karber [8].

Detection of VP5 protein by flow cytometry (FCM)

VP5 protein on the surface of bursal cells of chicken infected with virulent IBDV, a VP5⁻/IBDV-infected Vero monolayer and VP5-expressing Vero cells was analyzed by FCM. Briefly, twelve 30-day-old SPF Leghorn chickens were housed in negative-pressure isolator cages with HEPA-filtered air and inoculated intranasally with 50

containing pET-28a were used as controls. *M* prestained protein molecular weight marker (MBI Fermentas, USA); *C E. coli* BL21 (DE3) containing pET-28a

BLD₅₀ IBDV NB strain in a volume of 0.1 mL. Single-cell suspensions were produced from bursa of Fabricus (BF) tissues collected from infected chickens at 12, 24, 48 and 72 h p.i. as described previously [31]. Single-cell suspensions of Vero cell monolayers were prepared by digestion with 0.25% trypsin for 3 min, followed by three washes with PBS. Using the previously reported method [21], cells $(1 \times 10^6 \text{ cells})$ were first blocked in PBS containing 5% normal rabbit serum for 30 min at 4°C and then incubated with VP5 mAb and a secondary PE-labeled goat antimouse-IgG (SBA) on ice. Cells were analyzed using a



Fig. 4 Southern **a** and northern **b** blot analysis of monoclonal IBDV-VP5-expressing Vero cell lines. **a** Genomic DNA from monoclonal Vero cells expressing VP5 protein was analyzed by Southern blot using α -³²P-labelled VP5 probe. *Lane CK*+ contains a PCR-amplified IBDV-VP5 gene; *Lane CK*-contains genomic DNA from Vero cells transfected with the parental pCI-neo vector. *Lanes B1*, *C1*, *2F11*, *C5*, *D8*, *G10*, *C11* and *C12* contain genomic DNA from monoclonal IBDV-VP5-expressing Vero cell lines. **b** Genomic DNA from monoclonal Vero cells expressing VP5 protein was analyzed by northern blot using alpha-³²P-labelled VP5 probe. Lane CK- contains mRNA from Vero cells transfected with the parental pCI-neo vectors, *lanes B1*, *C1*, *2F11*, *C5*, *D8*, *G10*, *C11* and *C12* contain mRNA from Vero cells transfected with the parental pCI-neo vectors, *lanes B1*, *C1*, *2F11*, *C5*, *D8*, *G10*, *C11* and *C12* contain mRNA from vero cells transfected with the parental pCI-neo vectors, *lanes B1*, *C1*, *2F11*, *C5*, *D8*, *G10*, *C11* and *C12* contain mRNA from vero cells transfected with the parental pCI-neo vectors, *lanes B1*, *C1*, *2F11*, *C5*, *D8*, *G10*, *C11* and *C12* contain mRNA from vero cells transfected with the parental pCI-neo vectors, *lanes B1*, *C1*, *2F11*, *C5*, *D8*, *G10*, *C11* and *C12* contain mRNA from monoclonal cells expressing IBDV-VP5



Fig. 5 Expression of IBDV-VP5 protein in monoclonal cells, visualized by IFA. Using anti-VP5 mAb, the expressed VP5 protein was observed in the monoclonal IBDV-VP5-expressing Vero cells under a fluorescence microscope. *CK*-, Vero cells transfected with pCI-neo

vector; CK+ , Vero cells infected with IBDV; *B1*, *C1*, *2F11*, *C5*, *D8*, *G10*, *C11*, *C12* represent the eight strains of monoclonal IBDV-VP5expressing Vero cells. *Bar* 20 μ m (original magnification 40×)



Fig. 6 CEF cells infected by the VP5⁻/IBDV chimeric virus express VP4, but not VP5. Immunofluorescence staining was used to detect protein expression. a CEFs infected with VP5⁻/IBDV were stained with mouse anti-VP5 mAb. b CEFs infected with VP5⁻/IBDV were

stained with mouse anti-VP4 mAb. **c** Parental IBDV-infected CEF cells were stained with mouse anti-VP5 mAb. The nucleus was stained with 4',6-diamidino-2-phenylindole (*blue*). Bar 20 μ m (original magnification ×40)

FACSCaliburTM flow cytometer using CellquestTM software (BD, Pharmingen). Uninfected cells incubated with VP5 mAb and PE-conjugated mouse IgG1 (SBA) were used as parallel controls.

Results

Generation, characterization and reactivity of mAbs against VP5 protein of IBDV

BALB/c mice were immunized with the purified rVP5 protein expressed in *E. coli*. After cell fusion and screening, using the purified rVP5 protein as ELISA-coating antigen, eight hybridomas secreting mAbs to rVP5 were established and designated 1B2, 1B9, 2E6, 2E8, 3A4, 4B1, 4B7 and 5C2. As shown in Fig. 2, IFA analysis indicated that all eight mAbs recognized the VP5 protein within

IBDV-infected CEF (Fig. 2a) and Vero cells (Fig. 2b). Moreover, mock-infected CEF and Vero cells were not recognized by these mAbs. These analyses confirmed that the rVP5-derived mAbs could recognize the native VP5 protein of IBDV. Further western blot analysis demonstrated that all of the eight mAbs specifically recognized the purified rVP5 protein (Fig. 3), but none of these antibodies showed any reaction against cell lysate of *E. coli* BL21 (DE3) with pET-28a. Immunoglobulin subtype analysis indicated that the heavy and light chains of all mAbs were IgG1 and κ , respectively.

Generation of monoclonal Vero cells expressing VP5 protein of IBDV

After transfection with the pCI-neo-VP5 plasmid, the majority of Vero cells died by 7–8 days after inoculation in MEM containing $500 \mu g/mL$ G418. Surviving cells

remained under selection for 2-3 weeks and formed 25 visually distinct "cell islands". Independent G418-resistant cell islands were trypsinized and cloned by limiting dilution. After three rounds of selection, 13 G418-resistant clones were obtained and designated B1, C1, C5, C11, C12, D8, F4, F7, 2F11, G5, G10, H9 and H10. The DNA fragment of the VP5 gene was amplified from 8 of 13 monoclonal cells (B1, C1, C5, C11, C12, D8, 2F11 and G10). The eight PCR-positive strains were further subjected to Southern blot analysis to determine whether VP5 had been integrated into the genome. As shown in Fig. 4a, one to three copies of the VP5 gene were randomly inserted in the genome of each cell line. A northern blot assay (Fig. 4b) showed that all eight cell lines expressed mRNA corresponding to the VP5 gene. The cell lines C1 and C5 exhibited weak hybridization signals. Taken together, these data show that the VP5 gene of IBDV was integrated into the cellular genome and transcribed in Vero cells. Subsequently, VP5 protein expressed in monoclonal Vero cells were further investigated using IFA. In Fig. 5, it is shown that VP5 protein was detected in Vero cells after incubation with mouse anti-VP5 mAb. The monoclonal C5 strain was positive in Southern blot and northern blot analyses, but was negative for the detection of IFA at the 25th passage. A positive signal was detected in the other monoclonal strains with anti-VP5 mAb, indicating that 7 out of 8 stably expressed the VP5 protein of IBDV under G418 selection. In IFA analysis, it was obvious that the intensity of fluorescence differed among the monoclonal strains, and that VP5 protein was apparently expressed at higher levels in cell lines 2F11 and B1 than in the other five strains. Subsequent experiments were carried out using the 2F11 cell line generated here.

Generation of VP5⁻/IBDV chimeric infectious virus

To detect the expression of VP5 protein, CEF cells were infected with VP5⁻/IBDV. As expected, the VP4 protein was detected in the infected CEF cells by IFA with VP4-specific mAbs (Fig. 6b), but no VP5 protein was detectable with VP5-specific mAbs (Fig. 6a). However, VP5 protein was expressed in IBDV-infected CEF cells (Fig. 6c). These results indicate that the chimeric VP5⁻/IBDV infectious particles were generated and did not express VP5.

Replication and transmission of rescued VP5⁻/IBDV

When a CEF monolayer was inoculated with rescued VP5⁻/IBDV, VP5⁻/IBDV-infected cells began to exhibit a cytopathic effect (CPE) at 60 h.p.i. In contrast, a CEF monolayer inoculated with the parental IBDV exhibited CPE at 36 h.p.i. Correspondingly, the virus titers of CSC and supernatant in the IBDV-infected CEF monolayer were

more than 50-fold and 1,000-fold higher, respectively, than those of the VP5⁻/IBDV-infected CEF monolayer (Fig. 7a, b). However, the virus titer of the IBDV-infected CEF monolayer alone was 100-fold lower than that of the VP5⁻/IBDV-infected CEF monolayer (Fig. 7c). These



Fig. 7 Virus titer profile of cell supernatant and cell monolayer (*CSC*), cell supernatant alone and cell monolayer alone (*CM*) in VP5⁻/IBDV- and parental IBDV-infected cells. CEF cells were inoculated with equal amounts of the indicated viruses at 500 TCID₅₀, and progeny titer was determined by cytopathogenic effect (*CPE*). **a** Virus titer of CSC. **b** Virus titer of cell supernatant. **c** Virus titer of cell monolayer. Average titers and standard deviation (*error bars*) from two independent experiments are shown

Fig. 8 Flow cytometry analysis demonstrates that VP5 protein is expressed in IBDV-infected cells. VP5 expression in VP5⁻/IBDV- and IBDV-infected Vero cells VP5-transfected monoclonal Vero cells and bursal cells of IBDV-infected chickens was assayed by single-color flow cytometric analysis. Cells were stained as described with anti-VP5 mAb followed by PE-conjugated goat anti-mouse IgG. Mock-infected Vero cells served as negative controls (green line). The data shown are from a single representative experiment of three independent experiments)



results demonstrated that the absence of VP5 protein expression postpones and minimizes the cellular replication of IBDV and specifically inhibits the release of IBDV virions from infected cells.

Flow cytometric analysis of VP5 protein in infected cells

The expression of VP5 protein in VP5⁻/IBDV- and IBDVinfected Vero cells, VP5-transfected monoclonal Vero cells and bursal cells of IBDV-infected chickens was assessed by the FCM technique. As shown in Fig. 8, VP5⁻/IBDVand mock-infected Vero cells had no VP5 expressing (VP5⁺) populations. However, VP5⁺ cell populations were identified in VP5-transfected and parental IBDV-infected Vero cells. This confirmed that VP5 was detectable on the surface of IBDV-infected Vero cells. To further investigate whether VP5 protein molecules were present on the surface of bursal cells in IBDV-infected chickens, bursal singlecell suspensions were prepared at several different time points p.i. The data shown in Fig. 8 illustrate that the percentage of VP5⁺ cells was $4.5 \pm 0.5\%$ at 12 h p.i. and reached a peak $(34.0 \pm 1.6\%)$ at 72 h p.i. These data indicate that the number of VP5⁺ bursal cells increased gradually in IBDV-infected chickens during the first three days of IBDV infection.

Discussion

In the present study, we produced mAbs to the VP5 protein of IBDV using recombinant VP5 protein expressed in *E. coli*. These mAbs recognized native VP5 molecules expressed in IBDV-infected cells, confirming that they are capable of binding naturally expressed VP5 epitopes, and the mAbs provide an important tool for FCM of IBDV-infected cells.

Cell lines that express viral proteins are an important tool for understanding the protein's activity. Here, we obtained eight monoclonal Vero cell lines with one to three copies of the full-length VP5 gene inserted into the cellular genome, as shown by Southern blot (Fig. 4a). Subsequent IFA revealed that one of these monoclonal cell lines was not recognized by the anti-VP5 mAb, leading to the hypothesis that transcription of the VP5 gene is followed by post-transcriptional gene silencing (PTGS) in transfected cells. PTGS acts to degrade existing RNA and is a common defense mechanism against invasive nucleic acids in a broad range of organisms. It is also used to regulate controlled expression of endogenous genes [24]. We have previously demonstrated PTGS in transgenic potatoes [29, 30].

It is essential to understand cellular pathogenesis after virus infection in order to develop effective viral disease control. The formation of cellular lesions results from a series of interactions between viral components and virussensitive cells, and virus-induced cell death plays an important role in the pathogenesis of virus infection. A nonstructural protein of IBDV, VP5, is a highly conserved, basic, cysteine-rich class II membrane protein [11, 16] that has been shown to induce cell death or apoptosis when transiently overexpressed in BSC40, RP9 or CEF cells [11, 26]. In this study, however, seven monoclonal cell lines stably expressing VP5 under G418 selection did not undergo cell death or morphological changes, suggesting that the VP5 protein is not responsible for the induction of these processes. Moreover, in our experiments, VP5-protein-deficient IBDV was successfully recovered and produced a delay of cytopathic effect (CPE) in the infected cells, demonstrating that VP5 is not essential for viral replication but is relevant to the cytopathogenicity of IBDV. Instead, we observed that the virus titer is decreased in the supernatant of VP5⁻/IBDV-infected cells (Fig. 7b), indicating that the VP5 protein promotes the release of mature IBDV virions from infected cells, as suggested by Yao and Vakharia [26]. Besides, by rescuing the infectious clones of wild-type and VP5-deficient IBDV, Yao et al. [26] suggested that the VP5 protein of IBDV is involved in apoptosis of infected cells, but this was difficult to confirm in our monoclonal Vero cell lines stably expressing VP5 protein of IBDV, implying that the cytopathogenicity of IBDV-infected cells did not involve the VP5 protein of IBDV.

Generally, the lack of VP5 expression does not block intracellular virus production but does significantly hinder the release of infectious virus from infected cells. Using monoclonal Vero cell lines expressing the VP5 protein, it was confirmed that the VP5 protein is not responsible for the induction of cytopathogenicity. In addition, anti-IBDV VP5-specific mAbs have been generated as reagents for FCM analysis to finely map target cells.

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