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Binding chicken Anx2 is beneficial for infection with infectious bursal disease virus

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ABSTRACT

Infectious bursal disease virus (IBDV) causes a highly contagious disease in young chickens and leads to significant economic loss in the poultry industry. The identification of host cellular molecules that bind to IBDV will improve the understanding of the underlying pathogenic mechanisms. In this study, using a virus overlay protein-binding assay (VOPBA) and mass spectrometry (MS) analysis, IBDV was found to bind chicken Anx2, a membrane protein fraction from DF-1 cells. Its interactions were further confirmed by an overlay assay. The results of an immunofluorescence assay and flow cytometry showed that Anx2 could be expressed and colocalized with IBDV on the surface of infected cells. Moreover, either the soluble recombinant Anx2 or an anti-Anx2 antibody could inhibit IBDV binding to and infection of DF-1 cells in a dose-dependent manner. The knockdown of Anx2 of DF-1 cells by small interfering RNA clearly reduced the subsequent virus yield, and overexpression of Anx2 was capable of enhancing the virus yield. These results indicate, for the first time, that binding to Anx2 is beneficial for IBDV infection.

1. Introduction

Infectious bursal disease virus (IBDV), which is a member of the genus *Avibirnavirus* of the family *Birnaviridae*, is the causative agent of infectious bursal disease. IBDV is highly contagious and immunosuppressive among young chickens and can cause severe economic loss in the poultry industry (Müller et al., 2003). Two serotypes (serotype 1 and 2) of IBDV have been documented. Serotype 1 showed differing degrees of pathogenicity and mortality in chickens, while serotype 2 was avirulent. Blind-passage of wild-type IBDV in cell lines has been reported to generate cell culture-adapted IBDV with mutations in the capsid protein VP2 (Kwon and Kim, 2004; Lim et al., 1999; Mundt, 1999). After cell culture adaptation, IBDV can infect non-lymphoid cell lines, such as chicken embryo fibroblast (CEF) and DF-1 (immortal chicken embryo fibroblast) cells, but becomes attenuated in the chicken (Müller et al., 1986; Yamaguchi et al., 1996).

Binding of a virus to a specific receptor on the surface of susceptible target cells is the first event that is required for cell-tropism and effective infection. The identification of host cellular molecules that bind to a virus will improve the understanding of the life cycle of the virus and its pathogenic mechanisms. It has been reported that several host molecules are involved in IBDV binding to target cells (Gimenez et al., 2015; Yip et al., 2012). Nieper and Müller (1996) first reported that two proteins with molecular masses of 40 and 46 kDa were responsible for both serotypes of IBDV binding to CEF cells. Later, three proteins with molecular masses of 70, 82, and 110 kDa on the plasma membrane of LSCC-BK3, which is an IBDV-permissive chicken B lymphoblastoid cell line, were also found to bind IBDV (Setiyono et al., 2001). However, these proteins have not been further characterized. Recently, several components of the putative cellular receptor complex of IBDV have been reported, including heat shock protein 90 (cHSP90) (Lin et al., 2007), integrin alpha 4 beta 1 (Delgui et al., 2009, 2013), and surface IgM (Luo et al., 2010). However, none of the single proteins mentioned above was identified to mediate IBDV infection to non-permissive cells. Thus, further studies are required to identify whether other putative co-receptors or cell surface molecules are involved in IBDV binding or entry to host cells.

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In the present study, we identified chicken Anx2 as a new partner that is involved in IBDV binding to DF-1 cells, which is beneficial for IBDV infection. The study contributes to our further understanding the pathogenic mechanism of IBDV.

2. Materials and methods

2.1. Cells, virus, and antibodies

DF-1 cells were cultured in Dulbecco's modified eagle medium (DMEM), which was supplemented with 100 U/mL penicillin, 100 g/mL streptomycin, and 10% fetal bovine serum (FBS) in a 5% CO_2 incubator. The Gt strain was used, which is a cell culture-adapted and highly attenuated strain of IBDV. The Anx2 (rAnx2) protein (catalog no. ab93005), which was used for the inhibition of infection, and the anti-Anx2 antibody, which was produced in a rabbit (catalog no. ab40943) and used for western blotting, flow cytometry, and confocal microscopy, were purchased from Abcam (Cambridge, UK). The anti-IBDV VP2 antibody was produced in a mouse and was used for the detection of the virus in all experiments.

2.2. Infection of DF-1 cells with the IBDV Gt strain

DF-1 cells were grown in monolayers and were infected with the IBDV Gt strain at a multiplicity of infection (MOI) of 0.1. At 48-h post-infection, cells were observed for cytopathic effects (CPEs) using an inverted microscope.

2.3. Purification of IBDV Gt

Monolayers of DF-1 cells were infected with the IBDV Gt strain at an MOI of 0.1. Cell cultures were harvested when a CPE was obvious at 48-h post-infection. After freezing and thawing three times, cellular debris was removed by centrifugation at 10,000 g for 10 min at 4 °C. The supernatant was loaded on a 25% (wt/vol) sucrose cushion in TNE (0.01 M Tris–HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.6) buffer and centrifuged at 32,000 rpm for 3 h at 4 °C in a SW32 rotor (Beckman). The pellets were then resuspended in TNE buffer and further purified by density gradient centrifugation in CsCl with a density of 1.33 g/cm^3. Ultracentrifugation was performed overnight at 35,000 rpm with a SW55 rotor (Beckman) at 4 °C. Finally, the fractions were collected for electron microscopy analysis. The protein concentration of purified IBDV Gt was determined using the Bradford protein assay reagent according to the manufacturer's instructions (Bio-Rad). The highly purified IBDV Gt was stored at −70 °C and then used for VOPBA and other assays.

2.4. Preparation of membrane proteins of DF-1 cells

Membrane proteins of DF-1 cells were extracted using a ProteoExtract® Transmembrane Protein Extraction Kit (TM-PEK, Novagen) according to the manufacturer's instructions. The protein concentration was determined using a Micro BCA™ Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. The membrane proteins were used for further VOPBA analyses.

2.5. VOPBA and western blot analysis

In total, 80–100 μg of DF-1 cell membrane proteins was subjected to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE) and transferred to nitrocellulose membranes using transfer buffer (50 mM Tris base, 40 mM glycine, and 20% methanol [vol/vol]) in a semi-dry Bio-Rad blot apparatus. The membrane was blocked for 2 h at room temperature (RT) with 5% non-fat milk in phosphate-buffered saline (PBS, pH 7.4), washed once with 1% non-fat milk in PBS, and finally washed with an overlay buffer (1% non-fat milk in PBS, 220 mM NaCl). The VOPBA was performed by incubating the nitrocellulose membrane overnight at 4 °C with highly purified IBDV Gt (20 μg total protein/ml) in overlay buffer. The membrane was washed three times with 0.05% Tween-20 in PBS (PBST) and incubated with the anti-IBDV VP2 antibody in PBST for 3 h at RT. The membrane was then incubated with HRP-conjugated anti-mouse IgG as a secondary antibody for 2 h at RT and visualized using a DAB kit (Tiangen).

2.6. MS protein identification

Protein bands were excised, digested with trypsin and spotted onto a MALDI plate. A peptide mass fingerprint measurement for each sample was determined using a MALDI-TOF mass spectrometer (4800 Plus MALDI TOF/TOF™ Analyzer) (Applied Biosystems, USA). The peptide mass fingerprints (MS/MS Ion Search) were compared with fingerprints that were available in the NCBI (National Institutes of Health, Bethesda MD) Gallus database using the MASCOT search engine (Matrix Science, London, United Kingdom).

2.7. Identification of the interaction between recombinant chicken Anx2 and IBDV

Total RNA was extracted from DF-1 cells using the TRIzol reagent according to the manufacturer's instructions. Full-length chicken Anx2 cDNA was generated by PCR using the primers: 5′-CTGGGATCATGTCTACTGTCCATGAAAT-3′ (forward) and 5′-CGACTCGATCAGTCCTCTCCACCACA-3′ (reverse). The cDNA products were verified by sequencing and then cloned into the pET30a expression vector. Full-length recombinant chicken Anx2 protein was prepared by growing E. coli BL21 harboring pET30a-Anx2. Recombinant chicken Anx2 protein was purified from the pellet of the cell lysate using the Ni-NTA purification system (Invitrogen) according to the manufacturers' instructions.

Recombinant chicken Anx2 proteins were loaded into four lanes and subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane; after washing and blocking, the membrane was cut into four strips for VOPBA. One strip was incubated with purified IBDV virions, and one strip was incubated with overlay buffer (without virus) as a control. These two strips were then further incubated with an anti-IBDV VP2 antibody and HRP-conjugated goat anti-mouse IgG for 1 h at RT and visualized using a DAB kit (Tiangen). To show the position of Anx2, the remaining two strips were incubated with an anti-Anx2 antibody and HRP-conjugated goat anti-rabbit IgG as a secondary antibody for 1 h at RT and visualized using a DAB kit (Tiangen).

2.8. Analysis of Anx2 on DF-1 cells by flow cytometry

DF-1 cells were trypsinized with 0.25% trypsin without EDTA. Then, approximately 1.0×10^4 cells were collected for each experiment, washed with fluorescence-activated cell sorter (FACS) buffer (10 mM HEPES, 1% FBS, 0.1% NaN_3, 1 × Hank's balanced salt solution [HBSS]), and blocked with 3% BSA (in FACS buffer) for 30 min. Next, cells were washed with FACS buffer, incubated with the anti-Anx2 antibody or rabbit IgG (catalog no. I5006, Sigma) at 37 °C for 1 h, washed with FACS buffer, and centrifuged at 300 g for 5 min. Cells were then resuspended in FITC-conjugated anti-rabbit IgG antibody for an additional 1 h at 37 °C, washed twice, resuspended in PBS, and analyzed using a FACSAria™ cell sorter (BD, America).

2.9. Confocal microscopy

In total, 70% of DF-1 cells, which were plated in a monolayer on a 20-mm glass bottom cell culture dish, were incubated with purified IBDV Gt (50 μg total protein/mL) for 1 h at 4 °C. Then, the cells were washed three times with cold PBS to remove the unbound virus, fixed with 4% paraformaldehyde for 20 min, and blocked with 1% bovine serum albumin (BSA) for 1 h. Next, the cells were incubated with anti-Anx2 and anti-VP2 antibodies for 1 h at 37 °C and then washed three times with PBST. The cells were stained with a FITC-conjugated anti-mouse IgG antibody and a TRITC-conjugated anti-rabbit IgG antibody. After washing, the cells were examined using a SP2 confocal system (Leica Microsystems, Germany).

2.10. Inhibition of IBDV infection

2.10.1. Inhibition by an anti-Anx2 antibody

The inhibition of infection assays were performed as described previously (Lin et al., 2007). Briefly, DF-1 cells were seeded in 24-well plates with DMEM, which was supplemented with 10% FBS for 5 h. The medium was then removed, and the DF-1 cells were incubated with an increasing concentration (from 0 to 40 μg/0.25 mL) of anti-Anx2 antibody or a control antibody (rabbit IgG, catalog no. I5006, Sigma) for 1 h at 4 °C. After removal of the media, the cells were infected with IBDV Gt at an MOI of 0.01 at 4 °C for 1 h and then washed three times with DMEM. Medium was then added to the infected cells, and the infection was allowed to proceed for another 72 h at 37 °C. After infection, the supernatant was collected and the viral infectivity titer was measured as $TCID_{50}$ per milliliter using the Reed-Muench formula. The mean values and standard deviations of the data obtained from three independent experiments were calculated.

Meanwhile, flow cytometry was used to perform competitive attachment inhibition experiments with IBDV directed by the anti-Anx2 antibody. DF-1 cells in 24-well plates were incubated with an anti-Anx2 antibody (40 μg/0.25 mL) or a control antibody (rabbit IgG, catalog no. I5006, Sigma) for 1 h at 4 °C. After washing three times with DMEM, the cells were infected with IBDV Gt at an MOI of 0.1 at 4 °C for 1 h. After washing three times, the DF-1 cells were trypsinized with 0.25% trypsin without EDTA. The cells were then washed three times with FACS buffer, incubated with the anti-VP2 antibody at 37 °C for 1 h, and then incubated with FITC-conjugated anti-mouse IgG antibody for an additional 1 h at 37 °C. The cells were analyzed using a BD FACSAria™ cell sorter.

2.10.2. Inhibition by soluble recombinant Anx2 (rAnx2)

IBDV Gt was incubated with increasing concentrations (from 0 to 4 μg/0.25 mL) of rAnx2 protein before infection. These Anx2-containing viral mixtures were added to infect DF-1 cells (at an MOI of 0.01) that were seeded in 24-well plates for 1 h. Similarly, cells were infected with IBDV Gt, which was preincubated with different concentrations of a purified irrelevant protein (Avian reovirus σC protein, prepared in our lab) as a control. Later, infected cells were washed three times with DMEM. Fresh medium was then added to the infected cells and the infection was allowed to proceed for 72 h at 37 °C. After infection, the supernatant was collected and the viral infectivity titer was measured as described above.

Meanwhile, flow cytometry was also used to perform the competitive attachment inhibition experiments of IBDV directed by rAnx2 protein (4 μg/0.25 mL). These rAnx2-containing viral mixtures were added to infect DF-1 cells (at an MOI of 0.1) that were seeded in 24-well plates for 1 h. The irrelevant protein (Avian reovirus σC protein, prepared in our lab) was used as a control. The cells were then detached and subsequently enumerated by flow cytometry as described above.

2.11. RNA interference assays

Small interfering RNAs (siRNAs) were synthesized and 4.0×10^5 DF-1 cells were seeded into 6-well plates. On the following day, the cells were transfected with 400 nM Anx2-specific siRNAs (siAnx2) or the control of non-targeting siRNA (siScr); the siRNA target sequences were CCGUGACAAGGUGAUUTT (siAnx2). After 48 h, the interference effects were analyzed by western blot, the cells were infected with IBDV at an MOI of 0.01 at 37 °C, and the supernatants were collected at 72 h post-infection (p.i.). Viral titers were determined as described above.

2.12. Overexpression of chicken Anx2

The cDNA of chicken Anx2 was cloned into a pCAGGS vector, and 3 μg of the recombinant plasmid pCAGGS-Anx2 or the empty plasmid pCAGGS were transfected into DF-1 cells. After 24 h, the overexpression effects were analyzed by western blot as described above, and then, the cells were infected with IBDV at an MOI of 0.01 at 37 °C and the supernatants were collected at 72 h post-infection (p.i.). Viral titers were determined as described above.

3. Results

3.1. Preparation of highly pure IBDV particles

To prepare IBDV for the virus overlay protein-binding assay (VOPBA) and other binding assays, the Gt strain was propagated in DF-1 cells and then purified by CsCl gradient centrifugation. Several virus bands were observed after equilibrium density centrifugation (Fig. 1A). To obtain highly pure IBDV particles, each band was collected and analyzed by electron microscopy. The band at the bottom (Fig. 1A) with highly pure IBDV particles (Fig. 1B) was collected and used for VOPBA and virus binding assays.

3.2. Identification of chicken Anx2 as an IBDV-binding protein

To search for potential IBDV binding proteins, the cell membrane proteins obtained from uninfected DF-1 cells were analyzed using SDS-PAGE, transferred onto a nitrocellulose membrane and subsequently used in VOPBA. Under high salt conditions (220 mM NaCl), the membrane was incubated with highly pure IBDV particles and then incubated with an anti-VP2 antibody. The data obtained indicated that IBDV virions attached to a component of DF-1 membranes with an apparent molecular mass of 38 kDa (Fig. 2A). No reaction was found in the control DF-1 cell membrane without incubation with IBDV virions (Fig. 2A). To identify this protein, the protein band in the gel was excised, and after alkylation and in-gel digestion with trypsin, the peptide mass was analyzed by mass spectrometry (MS). The MS data were compared with protein databases, and the results revealed that chicken annexin II (Anx2) was ranked first among the peptides that were obtained (Table 1). In addition, the molecular mass of Anx2 was similar to that of the protein detected on the nitrocellulose membranes (Fig. 2A).

To further corroborate the VOPBA results and validate the MS data, chicken Anx2 gene was amplified and cloned into pET-30a (+) and expressed in the prokaryotic expression system. The recombinant Anx2 was further purified with a Ni-NTA purification system (Invitrogen) (Supplementary Fig. 1). Then purified recombinant chicken Anx2 was used to capture virions by VOPBA. After exposure to IBDV or overlay buffer (without virus), the bound IBDV was detected by an anti-IBDV VP2 antibody (Fig. 2B, top left). No band was observed in the membrane treated with the overlay buffer control (Fig. 2B, top right). In addition, the membranes were incubated

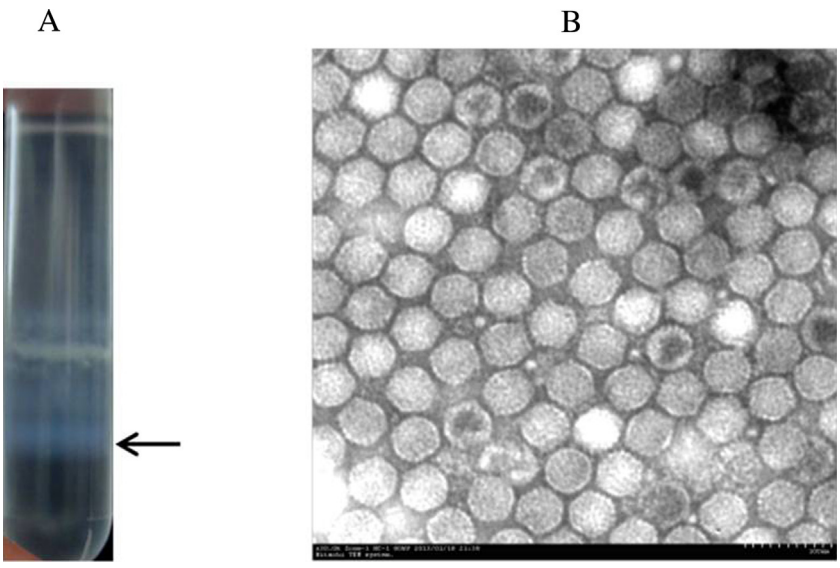

Fig. 1. Purity analysis of IBDV prepared by CsCl gradient centrifugation. (A) Several bands were observed after density centrifugation. The purity of each collected band was further analyzed by electron microscopy. The band on the bottom contains highly purified IBDV particles. (B) Electron micrograph with a 30,000× magnification of the band on the bottom with highly pure IBDV particles. Negative staining was performed with 2% uranyl acetate. Bar: 100 nm.

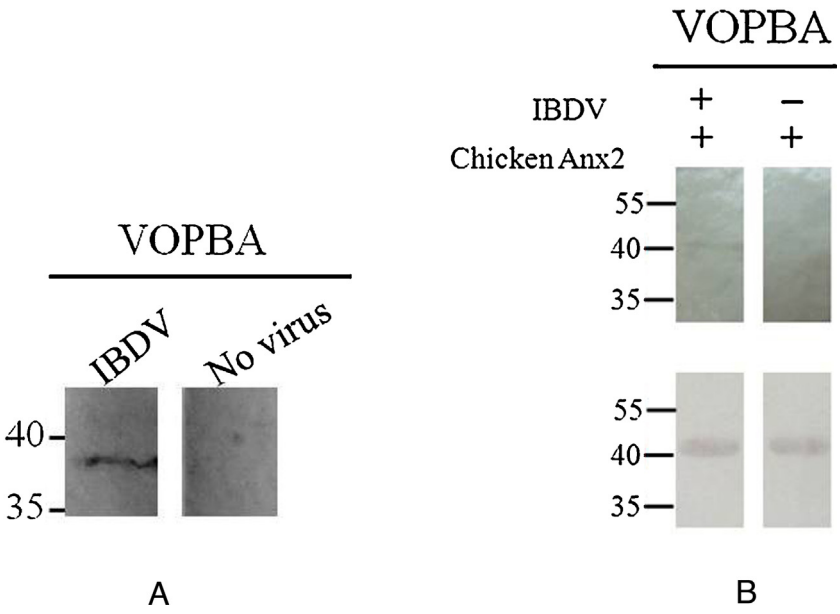

Fig. 2. Identification of an IBDV-binding protein Anx2. (A) VOPBA with membrane proteins of DF-1 cells. Membrane proteins that were obtained from DF-1 cells were subjected to SDS-10% PAGE, transferred to a nitrocellulose membrane, and incubated with 20 μg/mL of highly pure IBDV virions or a control without any virus. After incubation with the anti-VP2 antibody and immunoblotting with HRP-conjugated anti-mouse IgG, a protein band of approximately 38 kDa is visible in the presence of IBDV. There is no band in the control. (B) The recombinant chicken Anx2 proteins were loaded in four lanes, subjected to 10%SDS-PAGE and transferred to a nitrocellulose membrane; after washing and blocking, the membrane was cut into four strips for VOPBA. One strip was incubated with purified IBDV virions, and one strip was incubated with overlay buffer (without virus) as a control. These two strips were further incubated with an anti-IBDV VP2 antibody and HRP-conjugated goat anti-mouse IgG for 1 h at RT and visualized using a DAB kit (Tiangen). To show the position of Anx2, the remaining two strips were incubated with an anti-Anx2 antibody and HRP-conjugated goat anti-rabbit IgG as a secondary antibody for 1 h at RT and visualized using a DAB kit (Tiangen). Molecular size markers are indicated on the left (kDa).

with an anti-Anx2 antibody to confirm the co-localization of IBDV and Anx2 on the membrane (Fig. 2B, bottom).

3.3. The co-localization of IBDV with Anx2 on the cell surface

To confirm the binding of IBDV with Anx2 on the cell surface, the expression of Anx2 on the surface of DF-1 cells was first validated by flow cytometry. The positive signal was detected when DF-1 cells were incubated with anti-Anx2 antibody, but no signal was detected when DF-1 cells were incubated with the control antibody (Fig. 3A). To validate the co-localization of IBDV and Anx2, the DF-1 cells that were bound with or without virus were incubated with anti-VP2 and anti-Anx2 antibodies and then stained with a FITC-conjugated anti-mouse IgG antibody (green) and TRITC-conjugated anti-rabbit IgG antibody (red). The subcellular localization of IBDV and Anx2 was examined by fluorescence confocal microscopy. The results revealed that IBDV could bind DF-1 cell surfaces in which the partial co-localization of IBDV and Anx2 (yellow) were observed (Fig. 3B, top), while only the red fluorescence signal could be observed on the non-incubated cells (Fig. 3B, bottom).

Table 1
Identification of protein bands by mass spectrometry.

Rank	Accession No. in GenBank	Protein name	Protein MW (KDa)	Protein score
1	gi|45382533	Annexin II	38	93
2	gi|46048961	Glyceraldehyde-3-phosphate dehydrogenase	35	89
3	gi|211801	Glceraldehyde-3-phosphate dehydrogenase	35	51
4	gi|363743215	PREDICTED:UHRF1-binding protein 1	163	49
5	gi|363805615	Serine/threonine-protein kinase 10	113	48
6	gi|63401	Unnamed protein product	14	45
7	gi|118083014	PREDICTED:ublcarboxyl-terminal hydrolase 18	56	45
8	gi|363739093	PREDICTED:serine/threonine-protein kinase 10	107	42
9	gi|363734479	PREDICTED: pleckstrin homology domain-containing family H member 1	164	41
10	gi|363747010	PREDICTED: delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial-like, partial	14	40

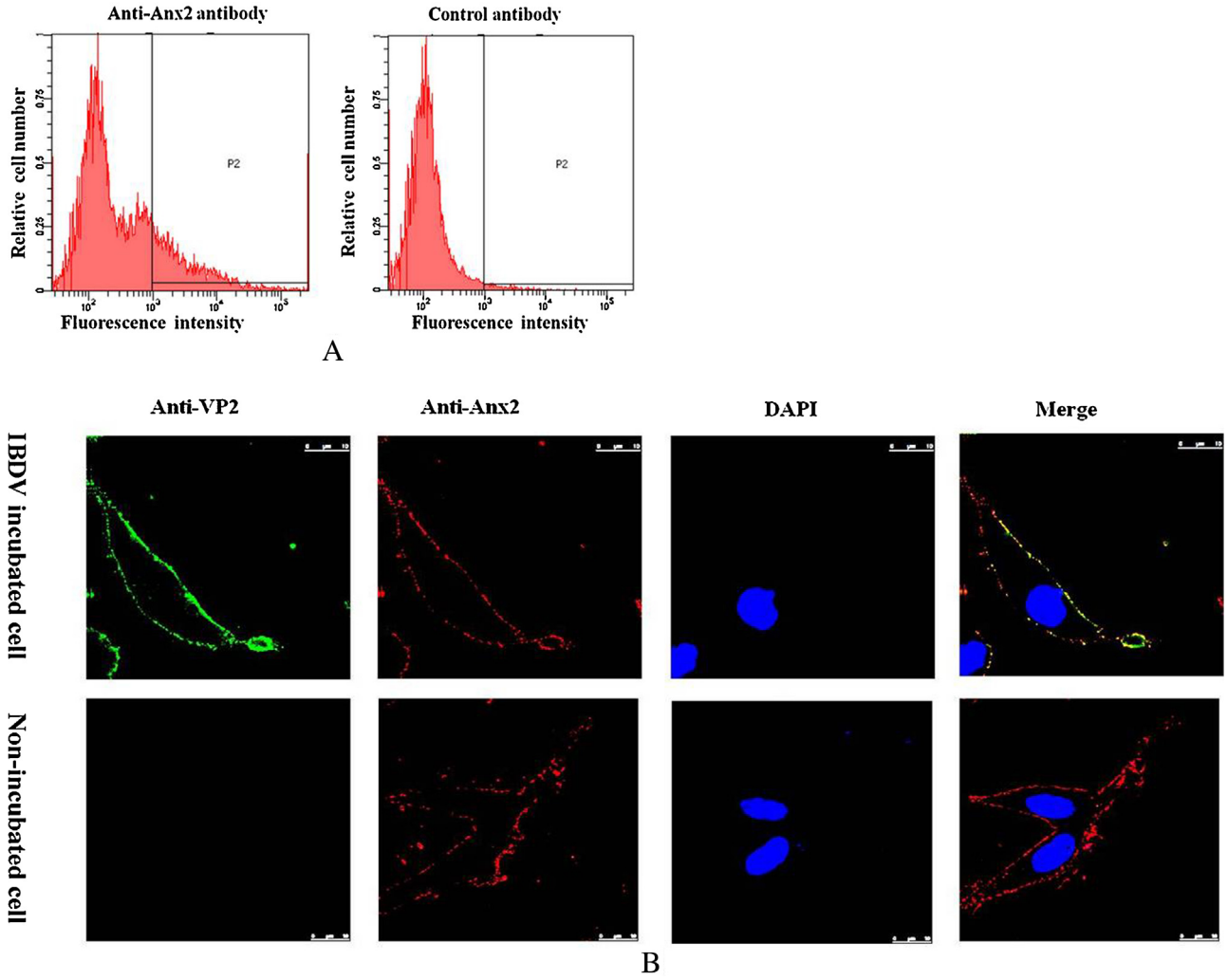

Fig. 3. Binding of IBDV to Anx2 on the surface of DF-1 cells. (A) Flow cytometric analysis of Anx2 expressed on the cell surface. DF-1 cells were incubated with an anti-Anx2 antibody or rabbit IgG (catalog no. I5006, Sigma) as a control antibody. After staining with an FITC-conjugated anti-rabbit antibody, the cells were analyzed by flow cytometry. The data shown are representative flow cytometry result. (B) The co-localization of IBDV and Anx2. DF-1 cells were incubated with purified IBDV Gt (top) or without IBDV as a control (bottom) for 1 h at 4 °C, washed three times with cold PBS to remove the unbound virus, fixed with 4% paraformaldehyde, incubated with anti-Anx2 and anti-VP2 antibodies, stained with an FITC-conjugated anti-mouse IgG antibody and an TRITC-conjugated anti-rabbit IgG antibody, and subjected to confocal microscopy analysis. The representative cells were viewed using confocal microscopy with IBDV (green) and Anx2 (red). Additionally, the position of the nucleus is indicated by DAPI (blue), and the three images are merged. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. IBDV infection was inhibited by an anti-Anx2 antibody and by rAnx2

To investigate the biological relevance of Anx2 in IBDV infection, an anti-Anx2 antibody was used to evaluate whether it could inhibit IBDV infection in DF-1 cells. The results showed that the anti-Anx2 antibody reduced the infectivity of IBDV Gt to DF-1 cells in a dose-dependent manner compared with the control, with up to a 1.0 log reduction in virus yield (Fig. 4A). Additionally, a soluble rAnx2 protein was used as a competitor in the infection assay. As shown in Fig. 4B, compared with the control, this rAnx2 was able to inhibit the infection of DF-1 cells by IBDV in a dose-dependent manner, and

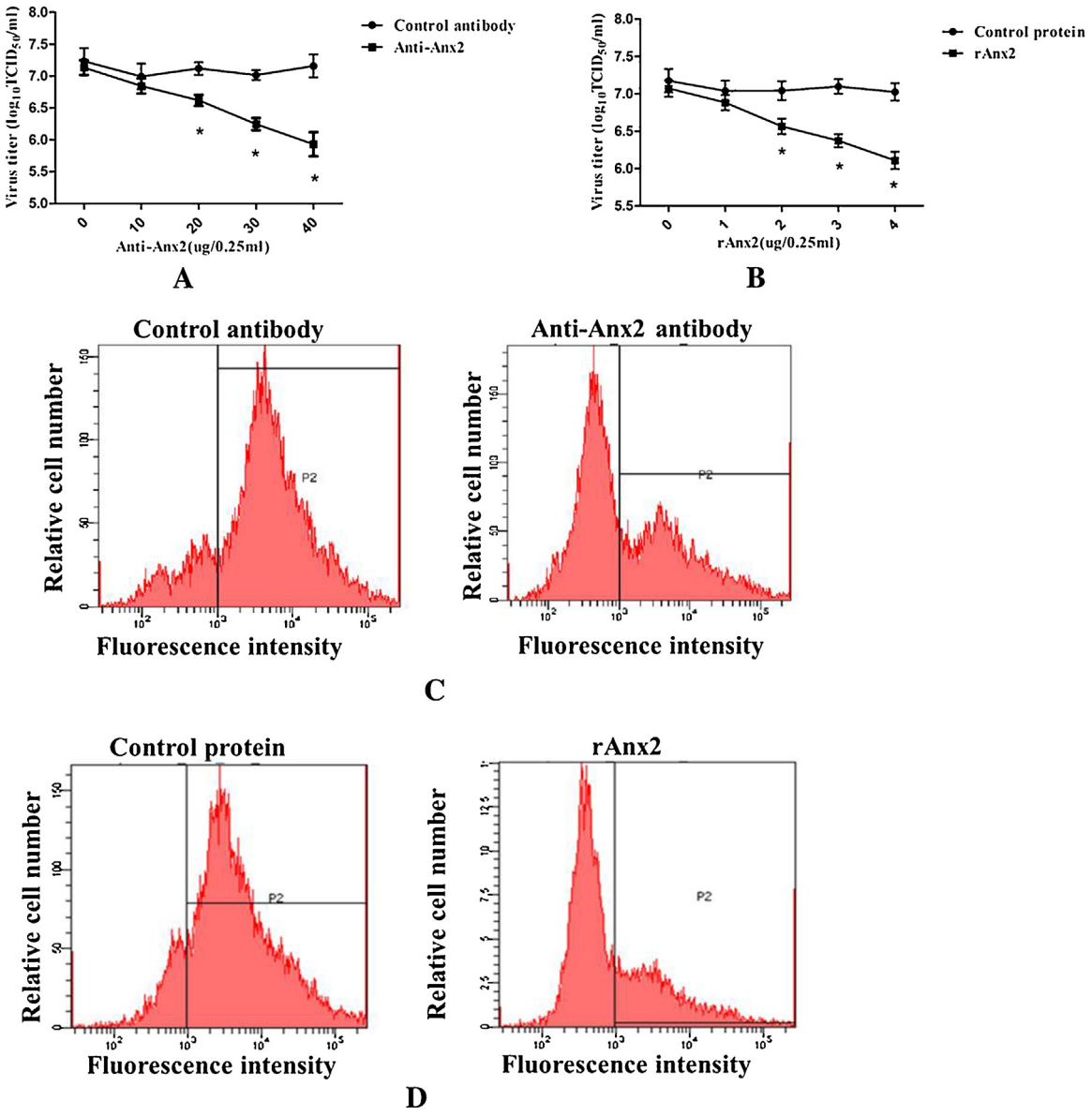

Fig. 4. Infection inhibition assays with an anti-Anx2 antibody or rAnx2 protein. (A) Infection inhibition assays with an anti-Anx2 antibody. DF-1 cells were preincubated with different concentrations of anti-Anx2 antibody or rabbit IgG (catalog no. I5006, Sigma) as a control antibody for 1 h at 4 °C. Subsequently, cells were infected with IBDV at an MOI of 0.01. Culture supernatants were collected at 72 h after infection, and the virus titer was determined. The results are presented as the mean ± SD for three independent experiments. (B) Infection inhibition assays with rAnx2. Different concentrations of rAnx2 or control protein (Avian reovirus σC protein, prepared in our lab) were preincubated with IBDV for 1 h at 37 °C before incubation with DF-1 cells (at an MOI of 0.01). Culture supernatants were collected at 72 h after infection, and the virus titer was determined. The results are presented as the mean ± SD for three independent experiments. Asterisks indicate significant differences compared with the control (*$P < 0.05$). (C) Binding inhibition assays with an anti-Anx2 antibody. Monolayers of DF-1 cells in 24-well plates were incubated with anti-Anx2 antibody (40 μg/0.25 mL) or a control antibody (rabbit IgG, Sigma) for 1 h at 4 °C. After the media were removed, the cells were infected with IBDV Gt at an MOI of 0. 1 at 4 °C for 1 h and then washed three times with DMEM. The DF-1 cells were trypsinized with 0.25% trypsin without EDTA. After incubation with an anti-VP2 antibody and staining with an FITC-conjugated anti-mouse antibody, approximately 1.0×10^4 cells were collected for flow cytometry. (D) Binding inhibition assays with rAnx2. IBDV Gt was incubated with the rAnx2 protein (4 μg/0.25 mL) before incubation. These Anx2-containing viral mixtures were added to infect DF-1 cells (at an MOI of 0.1) that were seeded in 24-well plates for 1 h at 4 °C. Similarly, cells were infected with IBDV Gt, which was preincubated with purified irrelevant protein (Avian reovirus σC protein) as a control. The cells were then detached and enumerated by flow cytometry as described in materials and methods. The data shown are representative flow cytometry analysis results.

a reduction of virus yields of approximately 1.0 log was observed at a concentration of 4 μg/0.25 mL rAnx2.

To further verify whether the binding of IBDV to DF-1 cells was affected by treatment with rAnx2 protein or an anti-Anx2 antibody, flow cytometry analysis was conducted. As shown in Fig. 4C and D, the number of positive cells significantly decreased after treatment with rAnx2 protein or anti-Anx2 antibody compared with the control. These data suggest that Anx2 was involved in the attachment and infection of IBDV to DF-1 cells.

3.5. Knockdown of Anx2 resulted in a reduction of virus production

To further test the relevance of Anx2 in IBDV replication, we examined viral production in DF-1 cells in which Anx2 expression had been inhibited using a siRNA method. DF-1 cells were treated with Anx2-specific siRNA (siAnx2) or a control of non-targeting scramble siRNA (siScr). The western blot results indicated that the levels of Anx2 expression were decreased by siAnx2, but not by siScr (Fig. 5A). The replication of IBDV in the DF-1 cells transfected

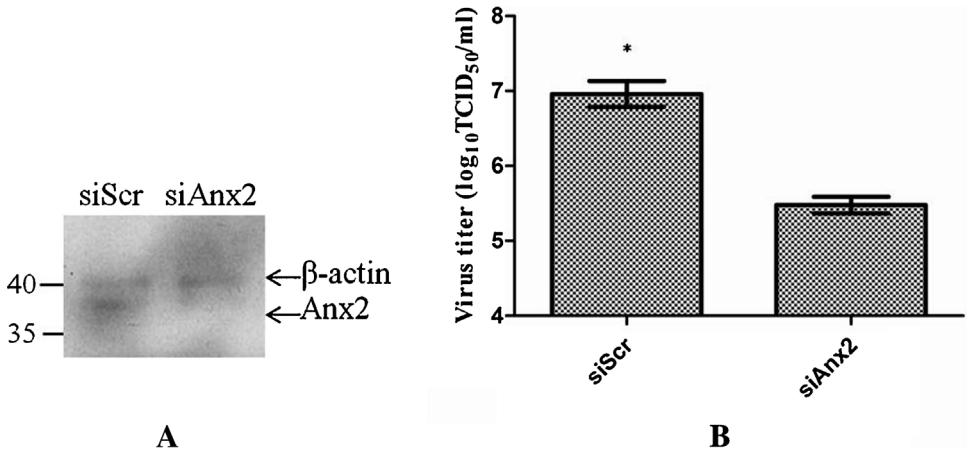

Fig. 5. Knockdown of Anx2 suppresses IBDV growth. (A) Knockdown of Anx2 protein expression levels by siRNA treatment. DF-1 cells were transfected with 400 nM siRNA against Anx2 (siAnx2) or with scramble siRNA (siScr) as a control. Cells were infected with IBDV at an MOI of 0.01 for 72 h, and then, the culture supernatants were collected and the viral titers were determined. In addition, the cell lysates were used to analyze the expression of Anx2 by SDS-PAGE and western blot. β-actin was used as a protein loading control. (B) IBDV titers in Anx2 knockdown cells. At 48-h post-transfection of siRNA, the cells were infected with IBDV at an MOI of 0.01, and the virus titers were determined in the supernatants collected at 72 h post-infection. The results are presented as the mean ± SD for three independent experiments. Asterisks indicate significant differences compared with the values of control siRNA treatment cells (*$P < 0.05$).

with siAnx2 or siScr was detected. The results indicated that siAnx2 treatment diminished the virus titer by up to 1.5 log at 72-h post-infection. The non-targeting siScr did not have a significant effect on the viral titer level (Fig. 5B).

3.6. Overexpression of Anx2 resulted in an enhancement of virus production

The plasmid pCAGGS-Anx2 or the control plasmid pCAGGS as transfected into DF-1 cells. After 24 h, the overexpression effects were analyzed by western blot. The data indicated that the levels of Anx2 expression were increased by transfection with pCAGGS-Anx2 (Fig. 6A). The replication of IBDV transfected with pCAGGS-Anx2 or pCAGGS was detected. The results indicated that the viral titers in the cells transfected with pCAGGS-Anx2 increased by 1.0 log at 72-h post-infection compared with the cells transfected with the control plasmid (Fig. 6B).

4. Discussion

The binding of a virus to susceptible cells is the first event required for a successful infection. However, a specific receptor on the surface of a susceptible host cell for the attachment of IBDV has still not been identified. To identify the binding molecules involved in IBDV infection, in the present study, cellular membrane proteins of DF-1 cells were screened by VOPBA and were further characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis. To reduce non-specific binding, VOPBA was performed with IBDV particles that were highly purified by density gradient centrifugation in CsCl and under high salt conditions (220 mM NaCl). The mass spectrum results revealed that chicken annexin II protein (Anx2) was ranked first among the peptides that were obtained, and the molecular mass of Anx2 (38 kDa) was similar to the protein that was detected on nitrocellulose membranes.

Annexin II (Anx2) belongs to the annexin family. In addition to its interaction with phospholipid membranes via its calcium ion-binding alpha-helical core domain (Rintala et al., 2008), it can interact with multiple cellular factors and is involved in the regulation of cellular functions, including endocytotic and exocytotic pathways, calcium-dependent F-actin filament bundling, and the profibrinolytic generation of plasmin (Donato and Russo-Marie, 1999; Gerke and Moss, 2002). Interestingly, previous reports showed that Anx2 is implicated in the infection of several viruses. For example, Anx2 was deeply involved in the infection of human cytomegalovirus (CMV) (Raynor et al., 1999; Wright et al., 1994, 1995), respiratory syncytial virus (RSV) (Malhotra et al., 2003), HIV (Ma et al., 2004; Ryzhova et al., 2006), hepatitis C virus (HCV) (Backes et al., 2001; Saxena et al., 2012), and Enterovirus71 (EV71) (Yang et al., 2011). In addition, it has been reported that Anx2 also contributes to some animal viruses. Anx2 is an interacting partner of porcine reproductive and respiratory syndrome virus (PRRSV) Nsp9 protein (Li et al., 2014) and can be incorporated into virions from PRRSV-infected cells (Zhang et al., 2010). However, the role of Anx2 in IBDV infection remains unknown.

Molecules that are involved in virus entry share some properties, such as binding with high affinity to the virus, surface location on the target cells, and inhibition of a viral infection when incubated with a specific antibody (Martínez-Barragán and Angel, 2001). First, the high-affinity binding of IBDV to Anx2 was verified by overlay assays under hypertonic conditions (Fig. 2B). Previous studies have demonstrated that Anx2 could be expressed on mammalian cell surfaces (Raynor et al., 1999; Malhotra et al., 2003; Backes et al., 2001). In this study, it was indicated that IBDV could bind DF-1 cell and partially co-localize with Anx2 expressed on the cell surface, which suggested that Anx2 might be involved in the attachment process of IBDV infection. To further confirm the speculation, two competitive attachment inhibition experiments directed by an anti-Anx2 antibody or rAnx2 protein were performed. The results showed that the anti-Anx2 antibody or rAnx2 protein not only competitively interfered with IBDV attachment to DF-1 cells but also significantly inhibited virus replication. Moreover, knockdown of Anx2 expression in DF-1 cells obviously decreased the titer of IBDV, while overexpression of Anx2 promoted IBDV replication. In addition, it has been reported that Ca^{2+} plays an important role in IBDV binding to permissive cells (Galloux et al., 2007; Yip et al., 2012). And Anx2 belongs to a family of Ca^{2+}-dependent phospholipid-binding proteins. We preliminarily explored whether Ca^{2+} was involved in the binding between Anx2 and IBDV. The data indicated that Ca^{2+} was involved in the distribution of Anx2 and the binding of IBDV to DF-1 cells (Supplementary Fig. 2). Taken together, these data confirmed that the binding to chicken Anx2 is beneficial for IBDV infection.

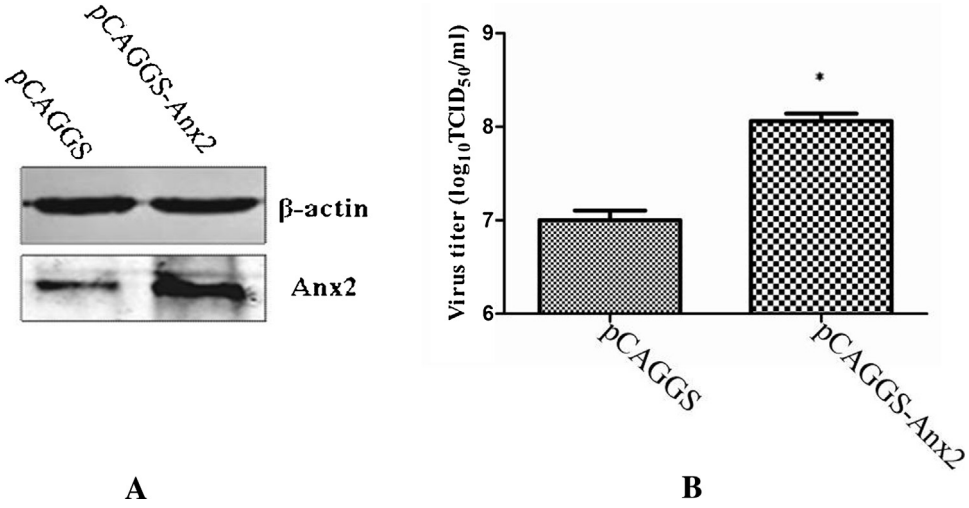

Fig. 6. Overexpression of Anx2 enhanced the virus production. Three micrograms of the recombinant plasmid pCAGGS-Anx2 or the empty plasmid pCAGGS were transfected into DF-1 cells. After 24 h, the overexpression effects were analyzed by western blot, with β-actin used as a protein loading control (Fig. 6A). The cells were then infected with IBDV at an MOI of 0.01 at 37 °C, and the supernatants were collected at 72 h post-infection (p.i.). Viral titers were determined (Fig. 6B). The results are presented as the means of three independent experiments. Asterisks indicate significant differences compared with the values of control cells (*$P < 0.05$).

In the viral infection inhibition experiments, we observed that IBDV infection could not be completely inhibited by the anti-Anx2 antibody, Anx2 protein, or siRNA. In previous studies, cHsp90 and alpha 4 beta 1 integrin were reported to be involved in IBDV infection. The authors of those studies also found that those proteins did not strongly influence IBDV infection (Lin et al., 2007; Delgui et al., 2009). Recently, researchers suspected that birnaviruses, including IBDV, might need to interact with several cell receptors during entry; some for attachment located at the top of the spike of VP2 and others for internalization located at the conserved groove of VP2 (Coulibaly et al., 2010; Lai et al., 2014). We speculate that Anx2 may serves as one component of the binding proteins for initial attachment, followed by interacting with cHsp90, alpha 4 beta 1 integrin, and probably, other cellular polypeptides required for IBDV binding and successful entry into the cell.

It is well known that wild-type IBDV replicates well in bursa target B-lymphoid cells but cannot adapt to CEF cells. Moreover, an attenuated strain can replicate in both cells, but the replication efficiency in B-lymphoid cells decreases. In our study, we also found that Anx2 could be expressed and co-localized with IBDV in B lymphocytes (data not shown). Anx2 most likely does not determine cell-tropism, but is likely involved in IBDV binding to host cells. The detailed mechanism deserves further study.

In conclusion, chicken Anx2 was identified as a new binding partner for IBDV. And the binding to Anx2 is beneficial for IBDV attachment and infection, which will contribute to our insight into the pathogenic mechanism of IBDV.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2015.07.024.

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