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Identification of the interaction and interaction domains of chicken anemia virus VP2 and VP3 proteins

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ABSTRACT

Chicken anemia virus (CAV) is a small, single-stranded DNA virus of *Anelloviridae* family. Its genome segments encode three proteins, VP1, VP2, and VP3. This study identified an interaction between VP2 and VP3 and mapped the interaction domains. Through the yeast two-hybrid (Y2H) system, VP2 was found to interact with VP3. The presence of the VP2–VP3 complex in CAV-infected chicken cells was confirmed by co-immunoprecipitation. Confocal microscopy showed that VP2 and VP3 were expressed in the cytoplasm in co-transfected Vero cells. In the Y2H system, the interaction domains were identified as being within the N-terminal aa 1–30 and C-terminal aa 17–60 for VP2 and the N-terminal aa 46–60 and C-terminal aa 1–7 for VP3. This study showed the interaction between VP2 and VP3 of CAV and identified multiple independent interactive domains within the two proteins. This provides novel information for investigating the biological functions of these proteins.

1. Introduction

Chicken anemia virus (CAV) is an important pathogen that causes severe anemia and immunosuppression in chickens, leading to serious economic losses worldwide in the poultry industry (Noteborn, 2004; Todd et al., 2001). The virus is a small, single-stranded DNA virus and has been classified into the *Anelloviridae* family. Its genome is about 2.3 kb in size, and there are three open reading frames (ORFs) present in the negative-sense genome encoding three proteins, VP1, VP2, and VP3 (Noteborn and Koch, 1995). VP1 is the major component responsible for assembly of the viral capsid. Despite the fact that the capsid contains only VP1, co-expression of VP2 is required for the induction of neutralizing antibodies in an *in vitro* expression system (Koch et al., 1995). VP2 is a dual-specificity protein phosphatase (DSP) that plays roles in viral replication, cytopathology, and virulence (Todd et al., 1990), and likely acts as scaffold protein during virion assembly (Peters et al., 2002; Lacorte et al., 2007) and induces apoptosis (Kaffashi et al., 2015). VP3, also referred as “apoptin”, has been shown to affect virus replication and virulence (Noteborn, 2004; Noteborn et al., 1994; Prasetyo et al., 2009; Wang et al., 2017). Interestingly, VP3 has attracted a great deal of interest due to its ability to induce apoptosis in multiple transformed and malignant mammalian cell lines without affecting primary and nontransformed cell, and now represents a promising candidate for antitumor therapy (Danen-Van Oorschot et al., 1997; Noteborn, 2005; Zhang et al., 2003).

Viral proteins generally function by interacting with viral and/or host cell proteins. Information about these interactions is thus essential for understanding viral biological processes. Previous studies have shown that co-expressed VP1 and VP2 proteins can react strongly with CAV-neutralizing antibodies *in vitro* and produced high titers of neutralizing antibodies to protect chickens from virus infection. However, VP1 or VP2 along did not induce a protective immune response (Koch et al., 1995; Noteborn et al., 1998). These studies suggested that the VP1–VP2 interaction is essential for the generation of neutralizing antibodies, which has is the basis for generating a highly effective CAV vaccine. However, whether the existence of other interactions between CAV proteins has yet to be demonstrated.

There is accumulating evidence showing that apoptin can interact with many cellular proteins. It was reported to bind to nuclear components, such as the homeodomain-interacting protein kinase 2 (HIPK2) (Poon et al., 2005), human death effector domain-associated factor (DEDAF) (DanenVan Oorschot et al., 2004), DNA (Leliveld et al., 2003), and APC-1, a subunit of the anaphase-promoting complex/cyclosome that is involved in the assembly and regulation of the

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cyclosome complex (Teodoro et al., 2004). Moreover, it was also found to regulate the activity of protein kinase C beta (PKC-β) activity in cancer cells through its interaction (Bullenkamp et al., 2015). These studies suggested that the interaction of apoptin with other proteins is essential for its biological function. However, little is known with regard to whether apoptin can interact with other CAV proteins. Given that VP2 and VP3 proteins are detected very early, *i.e.*, 12 h post infection, while VP1 is detected only after 30 h of infection (Douglas et al., 1995), we speculated that there may be an interaction between VP2 and VP3. Determining the nature of any interaction may help to understand the pathogenic mechanism of the virus because both the proteins are known to affect virus replication and virulence (Peters et al., 2002; Lacorte et al., 2007; Kaffashi et al., 2015; Noteborn et al., 1994; Prasetyo et al., 2009; Wang et al., 2017). The present study aimed to investigate whether VP3 can interact with VP2 using the yeast two-hybrid (Y2H) assay and co-immunoprecipitation (Co-IP) coupled with confocal laser scanning microscopy, and then to identify their tentative interaction domains by Y2H. Our results provide evidence of a VP2–VP3 interplay and show the presence of multiple independent interactive domains within the two proteins, which will provide novel insight for studying the biological functions of the proteins in CAV infections.

2. Materials and methods

2.1. Ethics statement

Care of laboratory animals and animal experimentation were performed in accordance with animal ethics guidelines and approved protocols. All animal studies were approved by the Animal Ethics Committee of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (SYXK (H) 2006-032).

2.2. Virus, cell lines, and antibodies

CAV strain M9905 was isolated and stored in our laboratory, and monoclonal mouse anti-VP2 and anti-VP3 antibodies were made by our group and stored in our laboratory. The plasmid pCAGGS was kindly provided by Dr. J. Miyazaki (University of Tokyo, Japan). Vero cells and MDCC-MSB1 cells, stored in our laboratory, were cultured in DMEM, supplemented with 10% FCS in a humidified incubator (Heraeus Instruments, Hanau, Hessen, Germany) in a 5% CO_2 atmosphere at 37 °C. Polyclonal rabbit anti-VP2 and anti-VP3 antibodies were prepared by immunizing rabbits with purified VP2 and VP3 proteins expressed in prokaryotes, respectively (unpublished data).

2.3. Construction of two-hybrid expression plasmids

Two-hybrid expression plasmids for full-length and truncated VP2 or VP3 were constructed with the primers shown in Table 1. The primers for truncated expression were designed based on the hydrophilic domains, antigenicity, and surface accessibility analysis predicted by DNAstar software (Madison, Wisconsin, USA). The full-length VP2 and VP3 of CAV were amplified by PCR using the specific primers BPVP2F/BPVP2R and BPVP3F/BPVP3R. N-terminal truncations of VP2 were amplified with primer pairs VP2ΔN30/BPVP2R, VP2ΔN35/BPVP2R, and VP2ΔN51/BPVP2R, and C-terminal truncations with primer pairs BPVP2F/VP2ΔC60 and BPVP2F/VP2ΔC17. N-terminal truncations of VP3 were amplified by primer pairs VP3ΔN45/BPVP3R, VP3ΔN60/BPVP3R, and VP3ΔN76/BPVP3R, and C-terminal truncations with primer pairs BPVP3F/VP3ΔC33 and BPVP3F/VP3ΔC7.

The PCR products were precipitated, digested with enzymes, gel-purified using the QIAEX-II method (Qiagen, Germany), and ligated with T4 ligase (Takara, Japan) into the yeast expression vectors $pDEST32_{DBD}$ and $pDEST22_{AD}$ (Clontech, Invitrogen, USA). Alternatively, the full-length VP2 was cloned into a pDEST32 vector to generate the bait plasmid pDEST32-VP2, and the full-length VP3 was cloned into a pDEST22 vector to generate the prey plasmid pDEST22-VP3. The ligation mixtures were transformed into *Escherichia coli* DH5α cells (Life Technologies, USA), which were subsequently grown with gentamicin or ampicillin selection. Plasmid DNA, prepared from several independent transformants, was screened for the presence of the insert. Positive clones were sequenced across the fusion junction by cycle sequencing with an ABI 310 sequencer to ensure the correct reading frames (primers 101, 102, and 103; Table 1).

2.4. Yeast two-hybrid screen

The ProQuest™ Two-Hybrid System (Invitrogen, Carlsbad, CA) was used to perform the Y2H screen in this study. All experiments were carried out according to the standard protocol for this system. The pDEST32-VP2, pDEST22-VP3, or other potential truncated interaction pairs were cotransformed into Mav203 yeast cells. Alternatively, the bait plasmids $pDEST32_{DBD}$-VP2, $pDEST32_{DBD}$-VP3, and empty prey plasmid $pDEST22_{AD}$ were also cotransformed into Mav203 cells to detect the self-activation profiles of the bait plasmids. In addition, co-transformed pEXP32/Krev1 and RalGDS-wt plasmids served as the positive control while pEXP32/Krev1 and pEXP22/RalGDS-m2 served as the negative control.

The transformed cells were grown on SD/-Leu/-Trp/-His medium containing 30 mM 3-Amino-1,2,4-triazole (3AT) for 5–7 days, and then the culture was continued on Master plates for 2 days. (The master plates were generated by streaking transformants that grow on SC-Leu-Trp-His + 3AT on SC-Leu-Trp plates to isolate single purified colonies). Clones were then transferred onto sterile nitrocellulose filter membranes, which were plated on YPAD plates to determine β-galactosidase activities using X-gal assay. Each enzyme activity assay was performed with at least five independent colonies, and β-galactosidase activities were calculated as described by Clontech. Alternatively, the yeast clones were transferred onto SD/-Leu/-Trp/-Ura solid media and cultured for 24 h. The clones were scrubbed using sterile flannelette for replica cleaning, culture was continued for 2 days, and the growth profile of the yeast colonies were observed.

2.5. Construction of eukaryotic expression plasmids

For studying the interaction between VP2 and VP3 *in vitro*, eukaryotic expression plasmids pCAGGS containing the complete ORF sequences of CAV VP2 and VP3 were constructed using the primer pairs 201/202 and 301/302 (Table 1). In brief, PCR products of VP2 and VP3 were amplified from the CAV strain M9905, purified using the QIAEX-II method (Qiagen), digested with KpnI/XhoI (Takara), and then ligated into pCAGGS to obtain the recombinant eukaryotic expression vectors pCAGG-VP2 and pCAGG-VP3. After screening by restriction digestion and PCR, the recombinant eukaryotic plasmids were sequenced with the plasmid-specific forward and reverse primers 104 and 105.

2.6. Confocal microscopic localization of VP2 and VP3 in Vero cells

Vero cells were transfected with pCAGG-VP2, pCAGG-VP3, and pCAGG-VP2 plus pCAGG-VP3 using Lipofectamine™2000 (Invitrogen, Carlsbad, CA) and cultured for 48 h. Immunostaining was carried out as follows. In brief, cells were washed with PBS before fixation for 20 min in 4% (w/v) paraformaldehyde and were subsequently permeabilized in 0.25% Triton X-100 in PBS and blocked with PBST with 5% (w/v) bovine serum albumin before immunostaining. For VP2 localization, cells were stained using anti-VP2 rabbit polyclonal antibodies (1:100) and FITC-conjugated goat anti-rabbit IgG secondary antibody (1:100) (Sigma-Aldrich, Merck KGaA, USA). For VP3 localization, cells were stained using monoclonal anti-VP3 (mAb) serum (1:100) (Santa Cruz, Los Angeles, CA) and TRITC-conjugated goat anti-mouse IgG secondary antibody (1:100) (Sigma-Aldrich). The cell nuclei were counterstained