

The Nucleocapsid Protein of Avian Infectious Bronchitis Virus Interacts with Chicken Ubiquitin-Conjugating Enzyme 9 (cUBC9)

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Abstract: Avian Infectious Bronchitis Virus (IBV) is an avian coronavirus. The Nucleocapsid (N) protein of IBV (IBV-N) is an important structural and functional protein and plays an essential role throughout the virus life cycle. Some cellular proteins may be involved in these complicated processes, little is known about virus host interactions. In this study, host cell proteins that interact with N protein were sought by screening a chicken lung cDNA library using a yeast two-hybrid system assay. IBV-N was shown to interact specifically with chicken Ubiquitin-Conjugating enzyme 9 (cUBC9) which belongs to the ubiquitin-conjugating enzyme family and is an important enzyme in the sumoylation system. Furthermore, the interaction between IBV-N and cUBC9 was confirmed by coimmunoprecipitation. The results of mapping the cUBC9 binding region of IBV-N indicated that N151-230 fragment of IBV-N is responsible for the majority of the binding to cUBC9. This virus-host protein interaction may provide insights into the function of IBV-N and facilitate rational targeting of the viral nucleocapsid protein for the development of structure-based vaccines and antiviral compounds.

Key words: IBV, nucleocapsid protein, cUBC9, interaction, yeast two-hybrid, coimmunoprecipitation

INTRODUCTION

Infectious Bronchitis Virus (IBV) causes an acute and contagious disease in domestic fowl with a significant impact on the poultry industry worldwide. It can replicate at many epithelial surfaces of the respiratory tract, alimentary tract, oviduct and kidney (Cavanagh, 2007). IBV is an enveloped positive-stranded RNA virus belonging to the family Coronaviridae and contains a genome of approximately 27 kb in length (Bourne et al., 1987) that encodes four major structural proteins including the Spike glycoprotein (S), Nucleocapsid protein (N), Membrane protein (M) and Envelope protein (E). The N protein of IBV (IBV-N), a basic, phosphorylated structural protein of 409 residues is highly conserved among IBV strains, especially within the middle region (Spencer et al., 2008).

Earlier studies have indicated that IBV-N plays an essential role in genome replication, transcription of subgenomic RNA, translation and formation of the nucleocapsid (Dove et al., 2006; Schelle et al., 2005; Zhao et al., 2004). However, details about the possible involvement of N protein in IBV pathogenesis are still unclear. The coronavirus N protein binds viral RNA to form a helical Ribonucleoprotein (RNP) that comprises the viral core structure (Chang et al., 2009; Hurst et al., 2005;

Ma et al., 2010; Saikatendu et al., 2007; Zhao et al., 2004), one of the key processes in the assembly of the virion and is essential for viability.

The N protein is the most abundant viral protein in coronaviruses and is produced throughout infection. Several functions have been postulated for the coronavirus N protein throughout the virus life cycle including viral packaging, viral core formation and signal transduction (Chang et al., 2009; Chen et al., 2009). It colocalizes with the replication complex in the sites of RNA synthesis (Hurst et al., 2005; Verheije et al., 2010). In addition, it has been shown that coronavirus N proteins may have RNA chaperone activity, binding RNA nonspecifically (Enjuanes et al., 2006; Zuniga et al., 2007). The RNA-binding (Fan et al., 2005; Grosseohme et al., 2009) and the oligomerization domains (Fan et al., 2005; Hurst et al., 2010) have been mapped. In particular, a serine-rich motif is probably important for the biological function of N protein and is conserved in all coronavirus N proteins (Tylor et al., 2009). Many studies have indicated that coronavirus N protein enters the host cell with the viral RNA and interferes with several cellular processes including induction of apoptosis and cell cycle arrest (Enjuanes et al., 2006) some of which involve interactions between N protein and host cell proteins (Surjit and Lal, 2008; Surjit et al., 2006) and may play a role in the pathology of coronavirus infections.

A yeast two-hybrid screen system was used to search for host cell proteins that may interact with IBV N protein in this study and it is demonstrated that the IBV N protein specifically interacts with chicken Ubiquitin-Conjugating enzyme 9 (cUBC9). cUBC9 belongs to the ubiquitin-conjugating enzyme family is involved in the process of protein sumoylation and is an important enzyme in the sumoylation system. This virus-host protein interaction may provide insights into understand better how IBV exploits its host.

MATERIALS AND METHODS

Virus and cell line: A nephro-pathogenic strain of IBV SAIBk was isolated from a field outbreak in Sichuan province in China. The SAIBK strain was propagated in the allantoic cavities of 10 days old SPF embryonated chicken eggs and the allantoic fluid was collected for viral isolation. The 50% chicken Infectious Dose (EID₅₀) was determined by inoculation serial 10 fold dilutions of virus into 10 days old SPF embryonated chicken eggs. Human embryonic kidney 293T cells (HEK 293T cells) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (vol/vol) Fetal Bovine Serum (FBS) and cultured in 5% CO₂ at 37°C. Transient transfection assays were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Plasmids and construction of recombinant vectors: The coding region of the *IBV-N* gene and the truncated mutants N1-150, N151-230, N231-330 and N331-409 were subcloned into the yeast two-hybrid vector pGBKT7. A cDNA library of chicken lung mRNA was used for screening. The cDNA library and SmaI-linearised pGADT7-Rec were cotransformed into yeast strain AH109. The *cUBC9* gene was amplified by PCR from the chicken lung cDNA library. For expression in mammalian cells, the *IBV-N* and *cUBC9* genes were cloned into pCMV-HA and pCMV-Myc, respectively with corresponding restriction enzyme cleavage sites. All of the vectors were purchased from American Clontech.

Yeast two-hybrid assays: The Matchmaker Library Construction and Screening kit (Clontech) was used to test the interactions between IBV-N and host cell proteins *in vitro*. The chicken lung cDNA library in the yeast plasmid pGADT7-Rec vector was screened in the yeast strain AH109. All procedures including incubation of the yeast, construction of a DNA-BD fusion vector, generation of the cDNA library, transformation, mating and screening followed the protocols provided by the manufacturer. To identify positive interactions, the

phenotypes of positive colonies were retested by restreaking on SD dropout plates 2-3 times and tested in a β -galactosidase activity assay as described in the users' manual. The cDNA inserts were rescued by plasmid isolation or PCR colony screening followed by sequencing. Finally, the interactions were retested in yeast by either cotransformation or mating.

Coimmunoprecipitation and immunoblot assay: To confirm the results observed with the yeast two-hybrid system, another independent assay and coimmunoprecipitation was carried out. HEK 293T cells were transiently co-transfected with pCMV-HA-N and pCMV-Myc-cUBC9. At 48 h posttransfection, the cells were harvested and washed with Phosphate-Buffered Saline (PBS) then lysed using cold mild lysis buffer. Cell lysates were clarified by centrifugation at 12,000 g for 20 min at 4°C and the supernatants were then immunoprecipitated with primary antibody bound to protein A/G-Sepharose beads (Oncogene, San Diego, CA, USA) and incubated overnight with shaking.

The beads were collected by centrifugation and washed five times with wash buffer. Eluted proteins were resuspended in 2×Sodium Dodecyl Sulfate (SDS) sample buffer and denatured for 5 min in boiling water before SDS-Polyacrylamide Gel Electrophoresis (PAGE) for protein separation then finally analyzed by Western blotting. For Western blotting, samples obtained from coimmunoprecipitation assays or transfected cell lysates were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were then incubated with anti-HA (HA-tag polyclonal antibody, Clontech) or anti-c-Myc (c-Myc monoclonal antibody, Clontech) antibody followed by horseradish peroxidase-conjugated rabbit anti-goat or anti-mouse antibodies, respectively. Binding was visualized by enhanced chemiluminescence.

RESULTS AND DISCUSSION

Identification of cUBC9 as a novel IBV-N interacting protein using the yeast two-hybrid system: The 3×10⁵ transformants were screened by mating and a total of 500 colonies grew up on Quadruple Drop-out (Leu-/Trp-/Ade-/His-) selection plates. Upon further screening, 26 positive pGADT7-cDNA plasmids were successfully isolated from yeast cells and rescued in *E. coli*. The cDNA insert from each plasmid was amplified by PCR with vector primers and digested with HaeIII and Sau3AI to distinguish different fragments.

The results of cotransformation of yeast AH109 with pGBKT7-N and positive pGADT7-cDNA plasmids as well as positive and negative controls. The β -galactosidase

activity of these clones was also assessed. In the filter assay the positive transformants were blue whereas false positives remain colorless or were a very light color (Fig. 1a). There were 9 confirmed positive clones and following sequence analysis showed that four were identified as chicken ubiquitin-conjugating enzyme 9 (cUBC9). As shown in Fig. 1b, the protein encoded by the pGADT7-cUBC9 clones interacted specifically with the IBV-N protein.

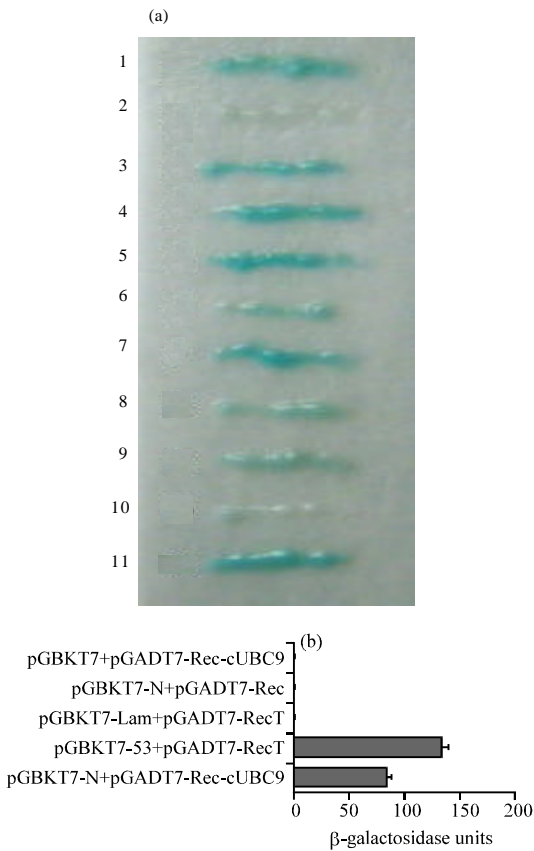


Fig. 1: β -galactosidase assay in yeast two-hybrid analysis showing positive and negative results. a) β -galactosidase filter assay. 1: Positive control. 2: Negative control. 3-11: eight randomly chose clones. 3, 4, 5, 7, 11 showing strong positive, in contrast, 6, 8, 9, 10 showing negative. b) Quantitation assay of β -examining the interaction between IBV-N and cUBC9. The plasmid pairs above were cotransfected into yeast strain AH109 and assessed for β -galactosidase activity. Results shown are the average units for triplicate assays. Error bars represent standard deviation

Cloning and sequence analysis of IBV-N and cUBC9: The *cUBC9* gene was cloned after PCR amplification from the

chicken lung cDNA library. The Open Reading Frame (ORF) of cUBC9 is 477 bp and encodes a polypeptide of 158 amino acids. The coding region of the *IBV-N* gene was amplified by PCR from pMD18-T-N.

The amino acid sequence of cUBC9 was aligned with 4 known sequences of UBC9 proteins in GenBank including those of humans, *Mus musculus*, *Drosophila melanogaster* and *Saccharomyces cerevisiae* (Fig. 2). The cUBC9 proteins were highly conserved, especially in higher eukaryotes which had identical amino acid sequences. The amino acid sequence of cUBC9 had about 85% identity with UBC9 of *Drosophila melanogaster* and only 56% identity with UBC9 of *Saccharomyces cerevisiae*. UBC9 is an important enzyme in the sumoylation system and many studies have indicated that the enzymatic active site is C93 amino acid residue (Duan *et al.*, 2009; Fan *et al.*, 2006) which is conserved in eukaryote (Fig. 2).

IBV-N has a lysine-rich region which has been predicted to be the Nuclear Localization Signal (NLS) in the C-terminal domain and a SR-rich region has been identified in the central domain that may be involved in RNA binding and protein-protein interactions. The results are shown in Fig. 3.

IBV-N physically associates with cUBC9 was revealed by coimmunoprecipitation:

The results of immunoblotting assay showed that similar amounts of HA-tagged IBV-N protein and c-Myc-tagged cUBC9 protein were expressed when cells were transfected with pCMV-HA-N and pCMV-Myc-cUBC9, either individually (Fig. 4a, Lanes 1 and 2) or in combination (Fig. 4a, Lane 3) which were detected by their corresponding antibody. The results of coimmunoprecipitation were shown in Fig. 4b. IBV-N fusion protein was detected by Immunoprecipitation (IP) using anti-c-Myc antibody and Western blot using anti-HA antibody (Fig. 4b, Lane 3). In addition, the cUBC9 fusion protein was detected by Immunoprecipitation (IP) using anti-HA antibody and Western blot using anti-c-Myc antibody (Fig. 4b, Lane 3). The specificity of IBV-N binding to cUBC9 was tested by cotransfection of the empty Myc vector with pCMV-HA-N and the empty HA vector with pCMV-Myc-cUBC9 (Fig. 4b, Lanes 1 and 2).

Mapping the cUBC9 binding region of IBV-N using the yeast two-hybrid system:

These four truncated N proteins were tested using the two-hybrid assay in yeast (Fig. 5b). The results indicated that the β -galactosidase activity of N151-230 fragment containing the SR-rich motif and AK²¹⁸AE is much higher than the activity of other

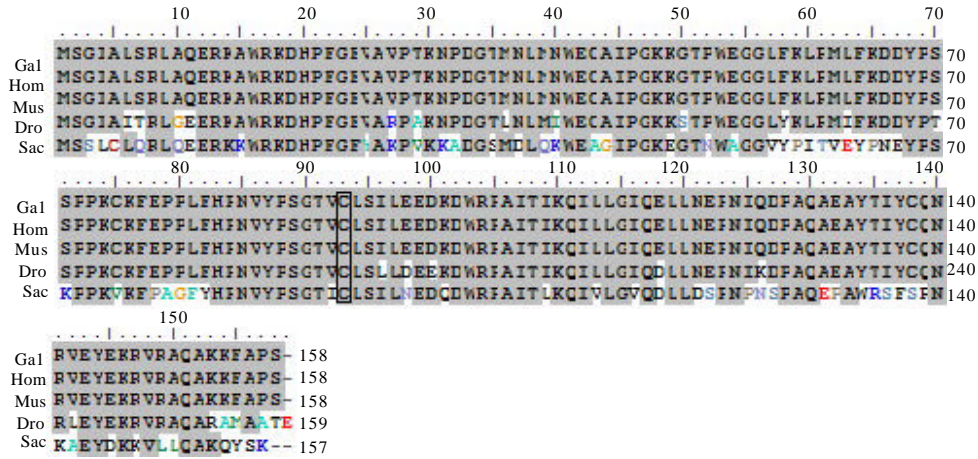


Fig. 2: The multiple alignments of UBC9 constructed by Clustal W. The source of the sequence: Chi-chicken GI: 19110799; Hom-*Homo sapiens* GI: 2597930; Mus-*Mus musculus* GI: 1943758; Dro-*Drosophila melanogaster* GI: 3892911; Sac-*Saccharomyces cerevisiae* GI: 1431070. Conserved residues are highlighted in gray. The enzyme active site C93 is conserved in the UBC9 which is boxed

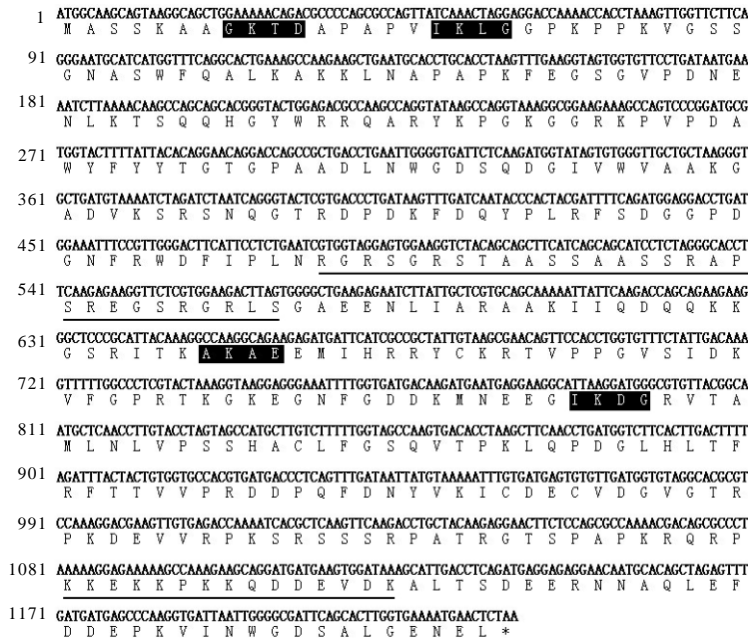


Fig. 3: Nucleic acid and amino acid sequences of IBV-N. Asterisk shows the termination codon. The sequences of SR-rich region and lysine-rich region are underlined and the consensus ψ KXE motif for sumoylation (where ψ is usually a hydrophobic residue and X is any amino acid) are marked with shaded background

fragments and which is responsible for the majority of the binding to cUBC9. Using the yeast two-hybrid screen, cUBC9 was identified as a potential host cell target of IBV-N. During viral infection many viral proteins are post-translationally modified by viral or cellular enzymes (Ahn *et al.*, 2001; Viswanathan *et al.*, 2010). Sumoylation is a post-translational modification that is involved in the

regulation of proteins of different cellular processes (Niedenthal, 2007). It is the process of covalent modification of cellular proteins by SUMO. UBC9 is an essential enzyme in the SUMO protein modification system and covalently conjugates SUMO-1 to target proteins and modulates various cellular processes.

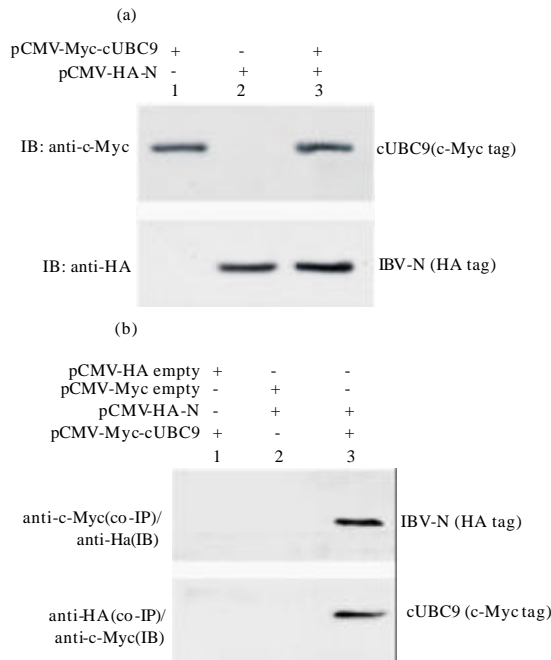


Fig. 4: Coimmunoprecipitation experiments for IBV-N and cUBC9 interaction; a) Immunoblotting; b) for IBV-N and cUBC9 were detected using anti-HA and anti-c-Myc, respectively to confirm the expression of the interested proteins. HA-tagged IBV-N protein and c-Myc-tagged cUBC9 protein were expressed respectively in HEK 293T cells. The plasmids used in lanes 1-3 were marked by plus and minus sign in the top; b) In coimmunoprecipitation (co-IP) for IBV-N and cUBC9, anti-c-Myc (or anti-HA) antibody was used for Immunoprecipitation (IP) and anti-HA (or anti-c-Myc) antibody was used for detection. Different combinations of the plasmids which were marked by plus and minus sign in the top of lanes 1-3 were used for cotransfection

The study suggests that sumoylation of IBV-N may occur. Earlier studies have shown that sumoylation modulates protein function through post-translational covalent attachment to lysine residues within targeted proteins (Johnson, 2004; Mo and Moschos, 2005; Muller *et al.*, 2004). A tetrapeptide ψ KXE (where ψ is usually a hydrophobic residue and X is any amino acid) has been defined as the consensus motif for sumoylation of target proteins containing an acceptor lysine residue (Seeler and Dejean, 2003). The nucleocapsid protein of some viruses can be sumoylated at this consensus acceptor lysine (Lee *et al.*, 2003; Maeda *et al.*, 2003).

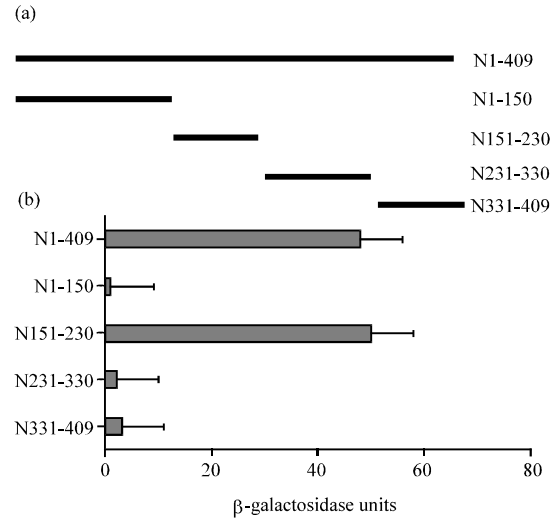


Fig. 5: Mapping the interaction domain of the IBV N protein; a) Schematic representation of the IBV N protein and the truncated mutants used in the yeast two-hybrid system; b) Quantitation assay of β -galactosidase activity was used to assess the binding ability of the truncated N protein mutants. The fragment N1 51-230 showed intensive binding to cUBC9. Results shown are the average units for triplicate assays. Error bars represent standard deviation

Researchers found three motifs, IK¹⁸LG, AK²¹⁸AE and IK²⁶⁴DG which are the consensus motif for sumoylation in the N protein (Fig. 3). In this study, the results showed that AK²¹⁸AE may be responsible for the majority of the binding to cUBC9. But studies on SARS-CoV have indicated that the consensus motif for sumoylation, GK⁶²EE in the N protein is not responsible for binding to hUBC9 (Fan *et al.*, 2006).

The RNA binding region in coronavirus N proteins overlaps with the binding site of UBC9 (Xu *et al.*, 2002). The NTD and CTD of IBV-N, encompassing residues 1-171 and 268-407, respectively interact with the noncoding region of the viral genomic RNA at its 3' end (Zhou and Collisson, 2000). In this study, N151-230 fragment of IBV-N is responsible for the majority of the binding to cUBC9. Thus, the interaction with cUBC9 may affect the specificity of viral RNA binding by IBV-N and might regulate the activity and function of IBV-N influencing virus assembly. Thus, cUBC9 was identified as a novel host cell target of IBV-N in chicken and this interaction can be predicted to play a crucial role in the regulation of various cellular processes during viral infection. Further studies will be required to investigate these possibilities and gain a fuller understanding of the

function of IBV-N and the potential pathogenic consequences of these virus-host protein interactions.

CONCLUSION

Researchers identified a novel host cell protein cUBC9 that interact specifically with N protein by screening a chicken lung cDNA library using a yeast two-hybrid system assay. cUBC9 belongs to the ubiquitin-conjugating enzyme family and is an important enzyme in the sumoylation system. Furthermore, the interaction between IBV-N and cUBC9 was confirmed by coimmunoprecipitation. Subsequently, the results of mapping the cUBC9 binding region of IBV-N indicated that N151-230 fragment of IBV-N containing the SR-rich motif and AK²¹⁸AE is responsible for the majority of the binding to cUBC9. This virus-host protein interaction may provide insights into the function of IBV-N during viral infection and understand better how IBV exploits its host and facilitate rational targeting of the viral nucleocapsid protein for the development of structure-based vaccines and antiviral compounds.

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