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Full Length Research Paper

Interaction of avian infectious bronchitis virus S1 protein with heat shock protein 47

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Infectious bronchitis (IB) caused by a coronavirus is an important disease in chickens; it mainly affects respiratory and kidney systems. The IBV-S1 protein is an important structural and functional protein. To identify the interaction between the IBV-S1 proteins and to elucidate the possible involvement of S1 protein in IBV pathogenesis, a chicken kidney cDNA library was screened using a yeast two-hybrid system assay. HSP47, a molecular chaperone protein facilitating the folding and assembly, was found to interact specifically with the S1 protein. The interaction between S1 and HSP47 was verified by co-localization experiment and co-immunoprecipitation of HeLa cell lysates expressing both proteins. The mapping studies localized the critical S1 sequences for this interaction to amino acids 340-470. Based on these results, we speculate that HSP47 is a functional target of infectious bronchitis virus S1 protein in cells. This is the first report demonstrating the interaction of HSP47 with a structural protein of plus-strand RNA viruses, indicating a new drug target for IBV.

Key words: S1 protein, HSP47 protein, yeast two-hybrid, co-localization, co-immunoprecipitation, protein-protein interaction.

INTRODUCTION

Infectious bronchitis (IB) is one of the most economically significant diseases that bring extensive harm to poultry industry. In young chicks, respiratory disease or nephritis lead to mortality, reduced weight gain and condemnation at processing exclusion, whereas in laying age chickens, the disease is subclinical and results in reduced egg production and anomalous eggs (Hewson et al., 2009). The disease model is more complex and difficult to prevent with therapeutics, thereby making it an extreme detriment to the poultry industry. Thus, the research of new technologies to prevent and treat the infectious bronchitis disease has become an urgent task to many researchers. IBV belongs to the family *Coronaviridae* (Mase et al., 2009). It is a pleomorphic enveloped virus with club-shaped surface projections (spikes) on the surface of the virion and its genome consists of the single stranded positive-sense RNA genome of approximately 27 kilobases (Boursnell et al, 1987). Like other coronavirus, the virion contains four major structural proteins: the spike (S) glycoprotein, the membrane (M) glycoprotein, the envelope (E) glycoprotein and the nucleocapsid (N) protein (Mase et al., 2009; Stern and Sefton, 1982). The S glycoprotein of IBV is posttanslationally cleaved into N-terminal S1 and C-terminal S2 subunits (Niesters et al., 1987). The S1 glycoprotein forms the distal, bulbous part of the spike, and the S2 glycoprotein anchors the S1 glycoprotein to the viral membrane (Boursnell et al., 1987). The S1 protein has been shown to be responsible for attachment to host cell membranes (Flint et al., 1987). The S1 protein subunit is also involved in infectivity and induction of neutralizing, serotype specific, and hemagglutination inhibiting antibodies (Cavanagh et al, 1986; Flint et al, 1987; Koch et al, 1990). Therefore, the S1 protein plays a crucial role in IBV pathopoiesis.

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Abbreviations: HSP, Heat shock protein; **IB,** infectious bronchitis; **IBV-S1,** infectious bronchitis virus S1 protein; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **ACE2,** angiotensin-converting enzyme 2; **ONPG,** ortho-nitrophenyl-β-galactoside.

Viral infection involves a number of steps. Initial processes crucial for the viral life cycle include attachment of the virion to host cell surface receptor(s) followed by cell entry and genome delivery (Smith and Helenius, 2004). The virus releases its genome into the cell either directly through the plasma membrane after cell attachment or by fusion of the viral membrane with the endosome membrane after endocytosis (Smith and Helenius, 2004; White et al., 1990). However, all of these and other steps in viral–host interaction for infectious bronchitis viral pathogens are poorly defined, due in part to the complex structure of IBV. Better understanding of the molecular mechanism of viral pathogenesis may contribute significantly to the development of prophylactic, therapeutic, and diagnostic reagents as well as help in infection control. The S1 protein of IBV has been shown to be responsible for attachment to host cell membranes; it is the logical candidate for initial interaction with host cell (Enquist and Racaniello, 2004). We therefore use S1 protein as the bait to screen for interacting proteins from a SPF chicken kidney cDNA library by the yeast two-hybrid assay system. Using this approach in this study, it is demonstrated that the IBV- S1 protein specifically interacts with a heat shock protein (HSP47), an important molecular chaperone. The interaction between S1 and HSP47 was verified by co-immunoprecipotation. It is also shown that the residues 340-470 of S1 protein is contributing to this interaction and HSP47 protein may play an important role in IBV pathopoiesis.

MATERIALS AND METHODS

Strains, general techniques

The strain of *Saccharomyces cerevisiae* used in this study was AH109 from Clontech company. Yeast cells were cultured at 30℃ either in a complete YPD medium (1% yeast extract, 1% peptone, and 2% glucose) or in a synthetic defined (SD) medium supplemented with required essential nutrients. Plates contained 2% agar. Transformation of yeast cells was performed by the lithium acetate (Gietz et al., 1995). *Escherichia coli* JM109 was used for general cloning. DNA manipulation was performed according to established protocol (Sambrook et al., 2001).

Plasmids and construction of recombinant vectors

The full-length S1 gene of the IBV coronavirus (SAIBk, AY282542, isolated by Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province, School of Life Science, Sichuan University) was PCR-amplified from a genomic construct of clone, and cloned into the pMD18-T vector (Takara, China). The full-length S1 gene was subjected to DNA sequencing, and the inserts were verified against the corresponding region of the IBV coronavirus complete genome SAIBk. To identify the putative domain of amino acid sequence required for HSP47/IBV-S1 interaction, different fragments IBV-S1 of gene were prepared by polymerase chain reaction (PCR). The six truncated mutants S1-S6 (constructs and putative functional domains were shown in Figure 4A) were subcloned into the yeast two-hybrid vector pGBKT7. The HSP47 gene was obtained by PCR from the chicken kidney cDNA library, and subcloned into the yeast vector pGADT7. For mammalian cell expression, the full-length S1 gene and HSP47 were subcloned into the pCMV-myc vector (Clontech, USA) and pCMV–HA, and fluorescence vector pEGFP-N1and pDsRed-N1, respectively. All DNA manipulations were performed as described by Sambrook et al (2001). All constructs were verified by restriction digestion and sequencing.

Screening of the kidney cell cDNA library by the yeast two-hybrid system

Yeast two-hybrid experiments were performed as described in the Clontech manual for the MATCHMAKER GAL4 two-hybrid system and in the Clontech yeast protocols handbook (Clontech, USA). Total RNA was isolated from Specific pathogen-free (SPF) egg fowl (50 - 100 g body weight), obtained from an Experimental Animal Center of Sichuan University. The kidney total RNA was extracted with TRIzol (Invitrigen, USA) following the manufacturer's manual. mRNA was isolated using the Oligotex-dT mRNA Midi Kit (Qiagen, Germany). The expression cDNA library was made by the SMART method according to the BD Matchmaker™ Library Construction Screening Kit manipulation manual (Clontech, USA). The ds-cDNA was purified by BD CHROMA SPIN™ TE-400 COLUMN (Clontech, USA). The resulting cDNAs were cloned via the SmaI into the pGAD vector transformed into yeast AH109 cells. Yeast cells were transformed with the pGBK-S1 and pGAD-cDNA library by the lithium acetate-mediated method, plated in selective SC/-trp-leu-his-ade medium. The fresh growing clones were assayed for β-gal activity by replica plating the yeast transformants onto Whatman filter papers (U.K); the filters were snap-frozen in liquid nitrogen for 10 sec twice and incubated in a buffer containing 5-bromo-4-chloro 3-indolyl-β-D-galactopyranoside solution at 30℃ for 1 - 8 h. Positive interactions were detected by the appearance of blue clones. The liquid β-galactosidase activity was determined using the substrate ONPG as described standard Protocols Handbook (PT3024-1, Clontech, USA). Data for quantitative assays were collected for yeast cell number and are the mean ± S.E.M. of triplicate assays. Appropriate positive/negative controls and buffer blanks were used. The positive pGAD-cDNA plasmids were isolated from positive yeast transformants by culture in leucine-deficient medium, which resulted in spontaneous loss of the plasmid pGBK-S1 and transformed into *E. coli* JM109 for sequence analysis. Auto sequencing assay was performed in Takara Company (China) and the resulting sequence was analyzed in the database of EMBL\Gene Bank by the BLAST program.

Mammalian cell cultures

The HeLa cell was obtained from Dr. Yunyi Yao (School of Life Science, Sichuan University). All cells were cultured at 37℃ in 5% CO_2 in DMEM containing 1 g/l glucose, 2 mM glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 0.1 mg/ml streptomycin and 100 U penicillin, and 10% FBS (Invitrogen, USA).

In vivo co-immunoprecipitation and western blotting

To reaffirm the results observed from yeast two-hybrid assays, another independent assay, co-immunoprecipitation was carried out. HeLa cells were co-transfected with the plasmids expressing pCMV-myc-HSP47 and pCMA-HA-S1 using the lipofectamine™ transfaction reagent (Invitrogen, USA). At 48 h post-transfection, cells were washed with PBS and then lysed in lysis buffer. Cell lysate was then mixed with anti-HA magnetic microbeads for 30 min on ice. 100 µl of 10% suspension of protein A–Sepharose was then added to the samples. The mixture was allowed to shake for 1 h at

4℃, after following which the beads were washed four times in lysis buffer, and protein was eluted in 2×SDS dye by boiling the sample for 5 - 10 min. Samples were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked using 0.5% BSA in PBST for 1 h, and incubated overnight with anti-myc antibodies (1:1000; Clontech, USA). The blot was then washed three times in PBST, incubated with anti-mouse IgG HRPO for 1 h and washed three times in PBST and the proteins were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The HeLa cells transected with the pCMV-myc and pCMV-HA vectors were used as negative controls.

Subcellular localization

HeLa cells were grown on coverslips in a 6-well chamber and simultaneously transfected with the recombinant fluorescence plasmids pEGFP-S1 and pDsRed-HSP47. After 24 h transfection, the cells were washed with PBS three times and fixed in 4% paraformaldehyde for 20 min at room temperature. The coverslips were then washed with PBS and mounted. Intracellular localization of the S1 protein and HSP47 was observed under a Leica confocal microscope (Germany).

RESULT

Identification of HSP47 as an IBV-S1 interacting protein by yeast two-hybrid system

The IBV-S1 ORF was subcloned into plasmid pGBK to be expressed as a fusion protein with the DNA-binding domain of the Gal-4 protein. The newly constructed plasmid, pGBK-S1, verified by auto sequencing assay, was used to transform yeast AH109. Yeast cells containing pGBK-S1 were transformed with a fowl kidney cDNA library. Approximate 4.7×10^6 transformants were screened for His-Ade-Trp-Leu independent growth and blue colony formation in the β-gal assay. Thirty-two positive clones were obtained. As some AD-cDNA fusion products can activate reporter gene transcription without interacting with the BD-S1 fusion protein, this false-positive clone can be identified using the technique of segregation analysis. Only 4 of 32 clones survived all genetic tests and were considered to be genuine positive clone, DNA sequence analysis of the fragment revealed that the four cDNA fragments inserted have a high identity with four genes in the GeneBank database. One of these clones was identified as heat shock protein 47(HSP47), which encodes a 405-amino acid protein, As shown in Figure 1, the protein encoded by the pGAD-HSP47 clones interacted specifically with the S1 protein and did not interact with the unfused GAL4-BD protein expressed from the parental pGBKT7 vector.

Co-immunoprecipitation determined the interaction of the S1 protein and HSP47

To further examine the interaction of the S1 protein and HSP47, co-immunoprecipitation was performed. The IBV S1 protein was fused at the amino terminus with a Myc-tag, and HSP47 was fused at the carboxyl terminus with a HA tag. The two plasmids were cotransfected into vero cells and immunoprecipitated. The immunoprecipitated complexes were separated on SDS–PAGE, and analyzed by Western blot with anti-myc monoclonal antibodies. As shown in Figure 2, the Myc-fused S1 protein immunoprecipitates with HA-HSP47. However, it does not immunoprecipitate with HA alone and neither can myc alone immunoprecipitate with HA-HSP47. These experiments confirm the results shown with the yeast two-hybrid system that the IBV S1 protein can interact with HSP47 protein and indicate that interaction occurs in mammalian epithelial cells.

Co-localization of the S1 protein and HSP47

The localization patterns of the S1 protein and HSP47 were investigated in HeLa cells. pEGFP-S1 and pDsRed-HSP47 were transfected simultaneously into HeLa cells. As shown in Figure 3, HSP47 and S1 protein localized mainly in the cytoplasm. The combined results indicated that the S1 protein and HSP47 co-localized in the cytoplasm of HeLa cells.

Mapping the HSP47 Binding Region of the S1 Protein by yeast two-hybrid assays

To map the involved regions of S1 protein in the IBV–S1/HSP47 interaction, six truncated fragments of S1 protein were generated (Figure 3A). These fragments, designated from ΔS1 to ΔS6, were cloned into pGBKT7 vector, and then co-transformed respectively with pGADT7-HSP47 into the AH109 yeast cell. The liquid β-galactosidase activity was determined using the substrate ONPG as described standard Protocols Handbook (PT3024-1, Clontech, USA). As indicated in Figure 4B, the β-galactosidase activity containing ΔS1, Δ3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

S2 and ΔS4 showed viability compared to the negative control, while β-galactosidase activity involving ΔS3, □S5 and ΔS6, did not show viability; moreover, the β-galactosidase activity of ΔS4 (340 - 470) fragment is much higher than the activity of ΔS2 (340-539) fragment. These results imply that theΔS4 (340 -470) domain of the S1 protein is responsible for the majority of the binding to HSP47.

DISCUSSION

Previous studies have demonstrated that the S1 protein is responsible for different functions in the life cycle of coronavirus. The S1 protein is essential for induction of neutralizing, serotype specific, and hemagglutination inhibiting antibodies via direct or indirect protein-protein interaction (Cavanagh et al., 1986; Koch et al., 1990).

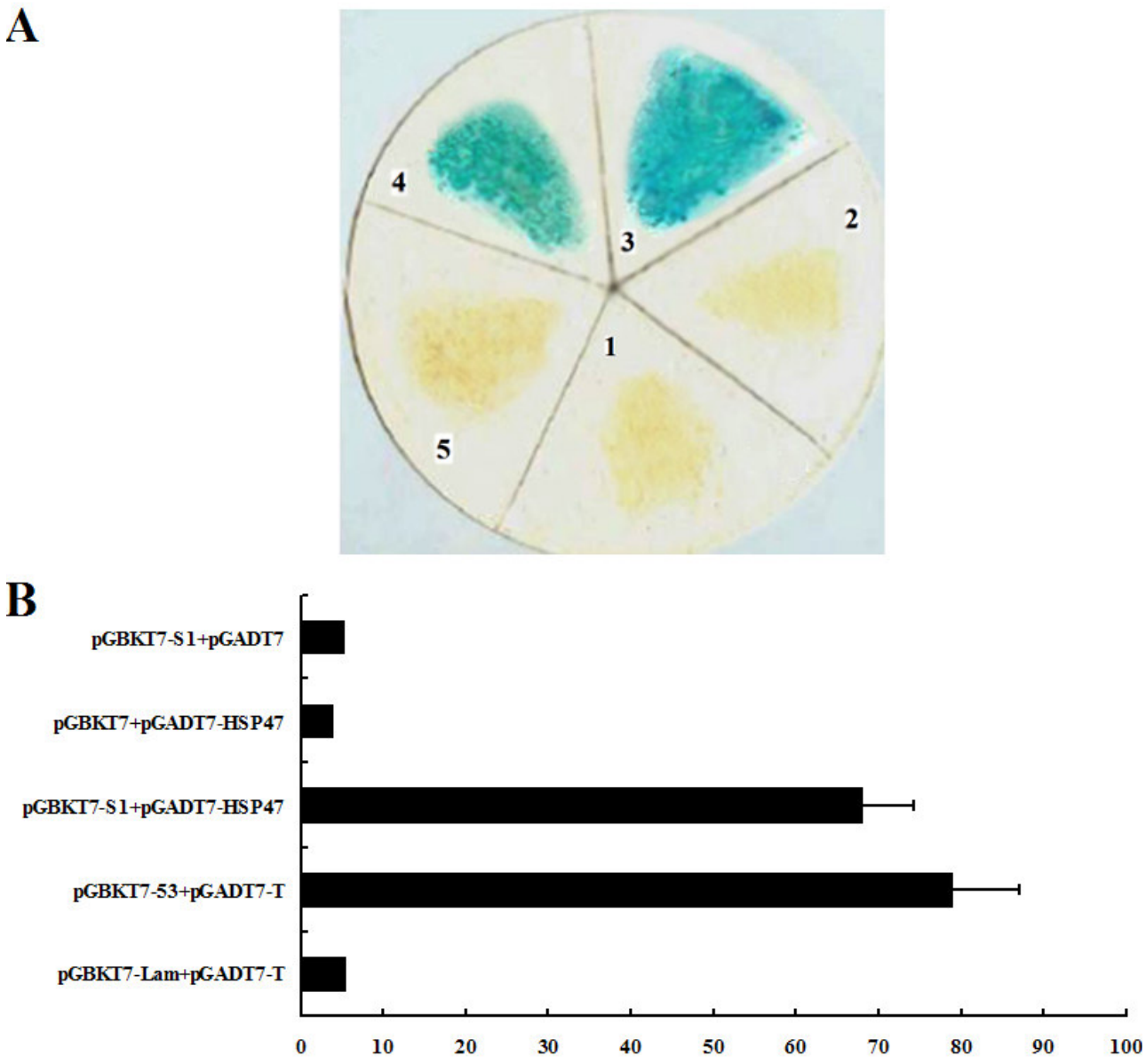

Figure 1. Yeast two-hybrid analysis of the IBV S1 protein and Hsp47 interaction. A: β-galactosidase filter assay. 1, Positive control pGBKT7-53+pGADT7-T; 2, negative control pGBKT7-Lam+pGADT7-T; 3, pGBKT7-S1 +pGADT7; 4, pGBKT7+pGADT7-HSP47; 5, pGBKT7-S1+pGADT7-HSP47. B: Yeast two-hybrid quantification of β-galactosidase activity. The plasmid pairs above were cotransfected into the yeast strain, and assessed for β-galactosidase activity. Results shown are the average units for triplicate assays. Error bars represent standard deviation.

Particularly, the S1 protein is suggested to perform a pivotal function in the attachment of coronavirus to a possible cell specific receptor (Enquist and Racaniello, 2004). Angiotensin-converting enzyme 2 (ACE2) as a cellular entry receptor of severe acute respiratory syndrome associated coronavirus by binding S1 protein has been reported (Li et al., 2003). Until now, there has been no reliable information on the receptor for IBV. Moreover, the host protein interaction S1 protein of IBV also was seldom reported.

In order to study the function of the S1 protein of IBV during the infection process, we searched for host proteins that interact with the S1 protein. Using a yeast two-hybrid system, we identified the chicken HSP47 protein as a candidate to interact with S1 protein. This interaction was confirmed *in vivo* by co-immunoprecipitate technique. The mapping studies localized the critical S1 sequences for this interaction to amino acids 340 - 470 by yeast two-hybrid assay. These results showed that the C-terminus of S1 could bind to HSP47 protein and the host protein HSP47 is a functional target of infectious bronchitis virus S1 protein in cells.

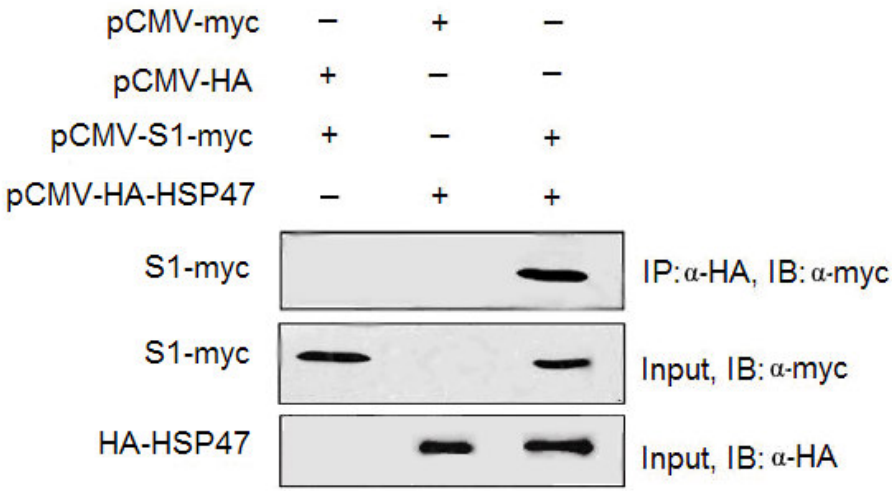

Figure 2. HSP47 protein immunoprecipitated with the IBV-S1 protein. Indicated plasmids were simultaneously transfected into HeLa cells. 24 h after transfection, co-immunoprecipitation was performed using anti-HA magnetic microbeads; the proteins immunoprecipitated (IP) were assayed with an anti-myc monoclonal antibody. Cell lysates were immunoblotted (IB) with anti-myc to confirm the expression of the object proteins.

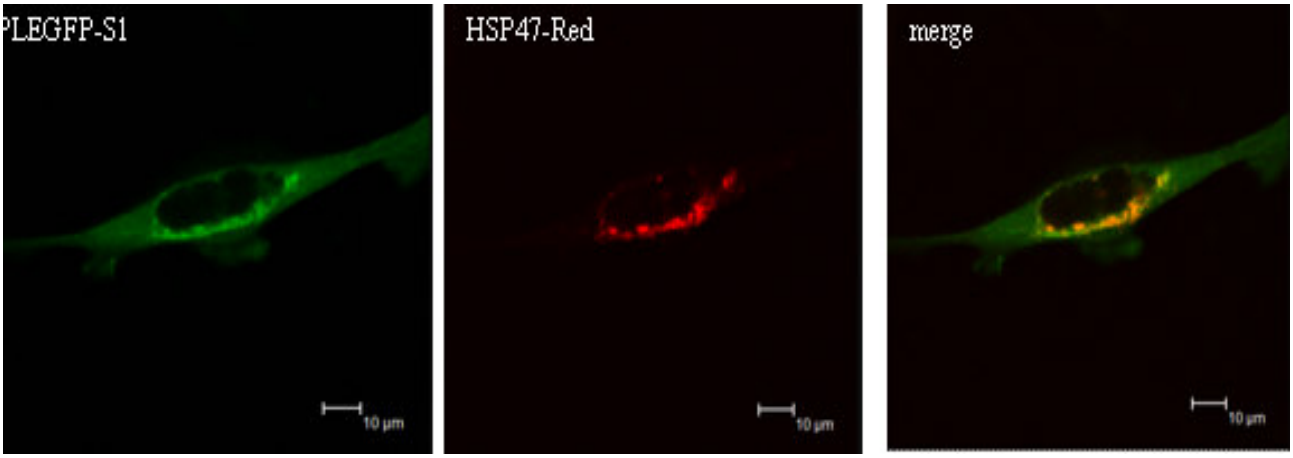

Figure 3. Co-localization of the IBV S1 protein and HSP47. pEGFP-S1 (green) and pDsRed-HSP47 (red) were co-transfected into HeLa cells. After 24 h, cells were fixed, mounted, and the localization of the proteins was observed with a Leica confocal microscope. As shown, the S1 protein and HSP7 were colocalized in the cytoplasm.

The interaction between S1 and HSP47 was verified in the present study. Heat shock proteins are molecular chaperones facilitating protein folding, assembly and intracellular transport, and thus are essential for cellular functions (Hightower, 1991; Nagata, 1996; Soti and Pal. 2005). Their synthesis is increased greatly in response to a variety of stressful stimuli. Among the HSP members, HSP47, a 47-kD glycoprotein, has the ability to function as a molecular chaperone in the endoplasmic reticulum (Nagata, 1996) and as a molecular chaperone-like function under stress conditions. Therefore, we presume that HSP47 plays a crucial role during the folding, maturation and secretion of S1 protein.

In conclusion, we have identified an interaction between S1, a spike protein and HSP47, an intracellular host protein. The knowledge of protein-protein interactions of the virulence factor S1 is of importance to understand the role of S1 during the infection process. However, the pathophysiological significance of the interaction between S1 and HSP47 is largely unknown; elucidation of these questions will depend on further studies of tissue distribution and cellular location after S1 binding to HSP47. Moreover, the disruption of interaction between S1 and HSP47 proteins using RNA interference technology may provide further clues to the specific function of S1 and HSP47 protein.

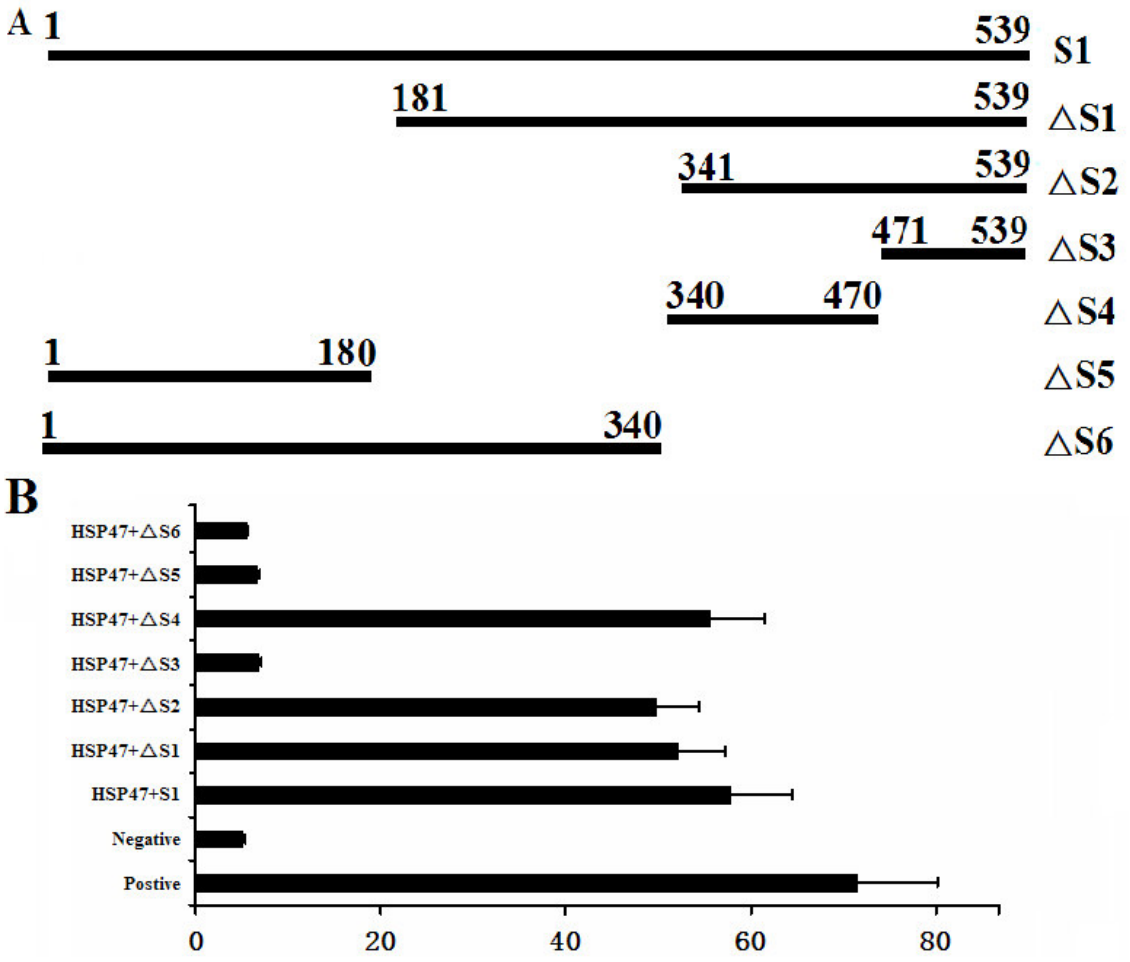

Figure 4. Mapping the interaction domain of IBV-S1. Schematic description of the truncated fragments (A) and the yeast two-hybrid assay results for IBV-S1/chicken HSP47 interactions in their truncated and non-truncated forms (B). The empty vectors pGBKT7 and pGADT7 co-transformed were used as the negative control and the pGBKT7-53 and pGADT7-T co-transformed were used as the postive control. Every experiment was repeated for at least three times and the data were obtained by average. The error bars represent standard error of the mean.

ACKNOWLEDGEMENTS

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