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Full Length Research Paper

Characterization of protein-protein interactions between the nucleocapsid protein and membrane protein of the avian infectious bronchitis virus

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Avian infectious bronchitis virus (IBV) is one of the major viral respiratory diseases of chickens. 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. Avian IBV belongs to the Coronaviridae and is similar to the other known coronaviruses. Previous studies have indicated that protein–protein interactions between nucleocapsid (N) and the membrane (M) proteins in coronavirus are related to coronavirus viral assembly. However, cases of IBV are seldom reported. In this study, yeast two-hybrid and co-immunoprecipitation techniques were applied to investigate possible interactions between IBV N and M proteins. We found that interaction of the N and M proteins took place *in vivo* and the residues 168 – 225 of the M protein and the residues 150 - 210 of the N protein were determined to be involved in their interaction. These results may provide some useful information on the molecular mechanism of IBV's N and M proteins, which will facilitate therapeutic strategies aiming at the disruption of the association between membrane and nucleocapsid proteins and indicate a new drug target for IBV.

Key words: Co-immunoprecipitation, membrane protein, nucleocapsid protein, protein-protein interaction, yeast two-hybrid.

INTRODUCTION

Avian infectious bronchitis virus (IBV), a group of three member of the genus Coronavirus, is a highly infectious pathogen of domestic fowl that replicates primarily in the respiratory tract as well as in epithelial cells of the gut, kidney and oviduct. It is of economic importance to the poultry industry due to the high morbidity and production losses associated with it. Clinical signs include: Tracheal rales, coughing, sneezing, poor weight gain and a decline in egg-shell quality and egg quantity. It may also cause the mixed infection of *Mycoplasma gallisepticum* and *Escherichia coli* as secondary infection and increase the mortality rate of poultry, causing a huge loss of poultry production (Yu et al., 2001; Liu and Kong, 2004; Alvarado et al., 2005).

The disease is virtually pandemic and has been discovered in North America, South America, Western Europe, Africa, China, Australia and New Zealand (Ignjatovic et al., 2006; Zhou et al., 2004; Bayry et al., 2005; Wang et al., 1997), where it has seriously endangered the world's poultry industry. The disease model is more complex and

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Abbreviations: IBV, Infectious bronchitis virus; **N,** nucleocapsid; **M,** membrane; **YPD,** yeast peptone-dextrose; **SD,** synthetic defined; **AD,** activation domain; **Trp,** tryptophan; **Leu,** leucine; **Ade,** adenine; **OPNG,** o-nitrophenyl β-D-galactopyranoside; **FBS,** fetal bovine serum; **SDS- PAGE,** sodium doedecyl sulfate-polyacrylamide gel electrophoresis; **BSA,** bovine serum albumi; **PBST,** phosphate buffered saline with tween 20; **ME,** membrane endodomain; **SARS,** severe acute respiratory syndrome.

difficult to prevent with therapeutics, thereby making it an extreme detriment to large-scale poultry production. Thus, the research of new technologies to prevent and treat the infectious bronchitis disease, and thereby reduce the enormous economic losses, becomes an urgent task to many researchers.

The etiologic agent of infectious bronchitis (IB) is IBV, an enveloped, positive-sense and single-stranded RNA virus that belongs to the coronaviridae family. Like other *coronavirus*, the coronaviridae contains four structural proteins: Nucleocapsid surrounding the viral RNA, an integral membrane glycoprotein, a small envelope protein and a spike glycoprotein located on the surface of the viral envelope. Nucleocapsid (N) protein is the composition of the internal nucleocapsid protein of IBV virus. It occupies 40% of viral protein consisting of 409 amino acids, and can be phosphorylated. N protein plays an important role in viral replication and assembly (It et al., 2001; Drees et al., 2001). The interaction of N protein and the sequence of pre-RNA contributes to the synthesis of viral mRNA and combines viral RNA to form a helical nucleocapsid binding. Several functions including viral packaging, viral core formation and signal transduction have been attributed to the coronavirus nucleocapsid (He et al., 2004; Hiscox et al., 2001).

Membrane (M) protein accounts for about 40% of total protein, consisting of 224 - 225 amino acids. IBV M protein was predicted to span the membrane three times and display a short ectodomain and a large endodomain (Figure 3B) on the basis of other coronavirus M proteins (Runtao et al., 2004; Kuo and Masters, 2002; De et al., 1998). It is believed that the C-terminal endodomain interacts with N and S proteins (De et al., 1998), which plays an important role in the formation as well as assembly of the virus. Previous study showed that CoV- M and N proteins could interact with each other and were of great significance in the assembly of the virus (He et al., 2004; Hiscox et al ., 2001). The protein-protein interaction between Coronaviridae N and M proteins has been reported on mouse hepatitis virus and transmissible gastroenteritis (Narayanan and Makino, 2001; Kuo and Masters, 2002). The study of SARS also proved that M protein could interact with the N protein (Runtao et al., 2004). However, the existence of such interaction in IBV has not been reported. In this research, the yeast two-hybrid and immunoprecipitation techniques were employed to study the interactions between M and N proteins in IBV, and elucidate the mechanism of molecular replication of IBV.

MATERIALS AND METHODS

Strains and general techniques

The strain of *Saccharomyces cerevisiae* used in this study was AH109 from Clontech (USA). Yeast cells were cultured at 30°C either in a complete yeast peptone-dextrose (YPD) medium (1% yeast extract, 1% peptone, 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 medium. *Escherichia coli* JM109 was used for general cloning. DNA manipulation was performed according to established protocol (Hiscox et al., 2001). Beta-galactosidase assays were carried out according to the Clontech matchmaker manual.

Plasmids and construction of recombinant vectors

The full-length N and M gene of the IBV (SAIBk, AY282542) was polymerase chain reaction (PCR)-amplified from a genomic construct of clone and cloned into the pMD18-T vector (Takara, China). The full-length N and M geneS were subjected to DNA sequencing, and the inserts were verified against the corresponding region of the IBV (SAIBk) complete genome. The full-length N gene was excised from the pMD18-T–N construct using the restriction enzymes EcoRI and BamHI, and ligated into the pGBKT7 vector to generate an N-terminal in-frame fusion with the GAL4 activation domain (BD). The full-length M gene was digested from the pMD18-T–M construct using the restriction enzymes EcoRI and PastI and ligated into the pGADT7 vector to generate an M-terminal in frame fusion with the GAL4 activation domain (AD). The full-length M gene was subcloned into the pCMV-HA vector (Clontech, USA), which carries HA tag. The full-length N gene was cloned into the pCMV-myc vector (Clontech,USA), which carries myc tag. The truncated mutants N1–149, N150–300, N301–409, N150–210 and N210–300 (constructs and putative functional domains are shown in Figure 3A) were subcloned into the yeast two-hybrid vector pGBKT7. The truncated mutants M1–29, M30–105, M106–225, M106–178 and M168–225 (constructs and putative functional domains are shown in Figure 3B) were subcloned into the yeast two-hybrid vector pGADT. All DNA manipulations were performed as described by Sambrook and Russell, (2001).

Yeast transformation and culture

Yeast two-hybrid experiments were conducted as described in the Clontech Manual for the MATCHMAKER GAL4 Two-Hybrid System and in the Clontech Yeast Protocols Handbook (Clontech, USA). The AH109 yeast strain was transformed with the appropriate plasmids, using the lithium acetate procedure and grown on SD plates in the absence of tryptophan (Trp) or leucine (Leu); (SD Trp− and SD Leu−). Protein interaction was tested on SD plates without Leu, Trp and adenine (Ade) (SD Leu− Trp− Ade−). After 48 – 96 h at 30°C, individual colonies were streaked out and tested for liquid and filter-lift β-galactosidase activity (Ober et al., 2002; Peiris et al., 2003; Pewe et al., 2005). The filter β-galactosidase assay, a parameter directly reflecting the strength of protein–protein interactions, was performed by streaking doubly transformed yeast colonies onto filter paper and allowing them to grow for 2 days on selection medium. Yeast was permeabilized by freezing yeast impregnated filters in liquid nitrogen and thawing at room temperature. This filter was placed over a second filter that was pre-soaked in Z buffer (pH 7.0).The liquid β-galactosidase activity was determined using the substrate o-nitrophenyl β-D-galacto-pyranoside (OPNG). Relative enzymatic activity was determined in five independent transformants. Data for quantitative assays were collected for yeast cell number and were the mean ± S.E.M of triplicate assays. Appropriate positive/negative controls and buffer blanks were used. All the yeast media were prepared according to the standard Protocols Handbook (PT3024-1, Clontech, USA).

Mammalian cell cultures

The vero cell was obtained from Doctor Yunyi Yao (School of Life

Science, Sichuan University). All cells were cultured at 37°C in 5% CO_2 in Dulbecco's modified eagle's medium (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% fetal bovine serum (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. Vero cells were co-transfected with the plasmids expressing pCMV-myc-N and pCMA-HA-M using the lipofectamine reagent (invitrogen). At 48 h post-transfection, cells were washed with phosphate buffered saline (PBS) and then lysed in lysis buffer. Cell lysate was 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°C, the beads were washed four times in lysis buffer, and protein was eluted in 2× sodium doedecyl sulfate (SDS) dye by boiling the sample for 5 - 10 min. Samples were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked using 0.5% bovine serum albumi (BSA) in phosphate buffered saline with tween 20 (PBST) for 2 h, and incubated overnight with anti-myc antibodies (1:1000; Clontech). 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. The vero cells that transfected pCMV-myc-N and pCMA-HA-M, separately, were used as negative controls.

RESULTS

Identification of the interaction between N and M protein by yeast two-hybrid system

To detect interaction between N and M proteins, a yeast two-hybrid technology based system was constructed. The recombinant plasmid PGBKT7-N contains the N coding sequence fused to the GAL4 DNA binding domain sequence, whereas, in PGADT7-M the M coding sequence was fused in-frame to the GAL4 activation domain sequence. Both constructs were co-transformed into AH109 and transformants were plated on medium lacking Trp, Leu and Ade (Figure 1A). Growth on Trp, Leu and Ade deficient medium demonstrates interaction of M and N proteins which results in reporter gene induction in the yeast strain. Moreover, the fact that the control yeast cells transformed with empty plasmids did not show cell growth (Figure 1A) suggested that this M/N interaction is specific in yeast. Accordingly, our yeast two-hybrid system was reliable and could be used for further research. To perform quantitative yeast two-hybrid tests between known proteins, beta-galactosidase assays were carried out. The interaction of N and M was obvious in the described data obtained in Figure 1B.

Co-immunoprecipitation verifies the interaction of M and N proteins

To examine further the interaction of the N and M proteins, coimmunoprecipitation was performed. Vero cells were co-transfected with amino-terminal HA-tagged M and carboxy-terminal myc-tagged N and immunoprecipitated with anti-HA magnetic microbeads, and washed extensively. The immunoprecipitated complexes were separated on SDS–PAGE and analyzed by western blot with anti-myc monoclonal antibodies. As shown in Figure 2, about 25 kD protein in accordance with M protein, was obtained from co-transfected cell, which showed that the myc-fused N protein immunoprecipitates with HA-M. Moreover, HA-M alone and myc-N alone could not be immunoprecipitated. These experiments confirm the results shown with the yeast two-hybrid system that the N protein can interact with M protein and indicate that interaction occurs in mammalian epithelial cells.

IBV M/N interaction region mapping by yeast two-hybrid assays

In order to identify the interaction region, N protein of the IBV was divided into three domains (Figure 3A). The three sequences were cloned respectively, and connected with the yeast vector, pGBKT7. Three deletion mutants of N protein were constructed and put together with the pGADT7-M of yeast strain AH109 at a temperature of 30°C. Four to five days later, the detection of beta-galactosidase activity shows that the middle domain of 150 - 300 amino acids interacts with M (Figure 3C). To further explore the binding fragment of M, N150-220 (including SR-rich region) and N210-300 were studied by the constructed yeast two-hybrid system. The result (Figure 3C) indicates that the N150-220 fragment contains the domain which mediates the interaction of N with M protein. M protein of IBV was divided into three domains (Figure 3B), according to the secondary structure of M protein. We found that the large M endodomain (ME) could interact with N protein by yeast two-hybrid assays (Figure 3C). To map the involved regions of ME in the IBV N/M interaction, It was found that amino acids 168 - 225, but not amino acids 106 - 178, could interact with N protein (Figure 3C). In summary, the residues 168 - 225 of IBV M and the residues 150 210 of IBV N protein were proved to be crucial for this interaction.

DISCUSSION

In this study, by yeast two-hybrid and immunoprecipitation techniques, we fully characterized IBV M/N interaction. It was found that the residues 168 – 225 of M protein and the residues 150 - 210 of N protein contributed to this interaction. Interaction between M and N in coronaviruses has been reported in literatures (; Kuo and Masters, 2002; De et al., 1998; Opstelten et al., 1995 Kelley et al., 2005). We found that the residues 168 - 225 of IBV M protein might contribute greatly to IBV M/N interaction, which seems to be compatible with the reported result that the M protein binds to

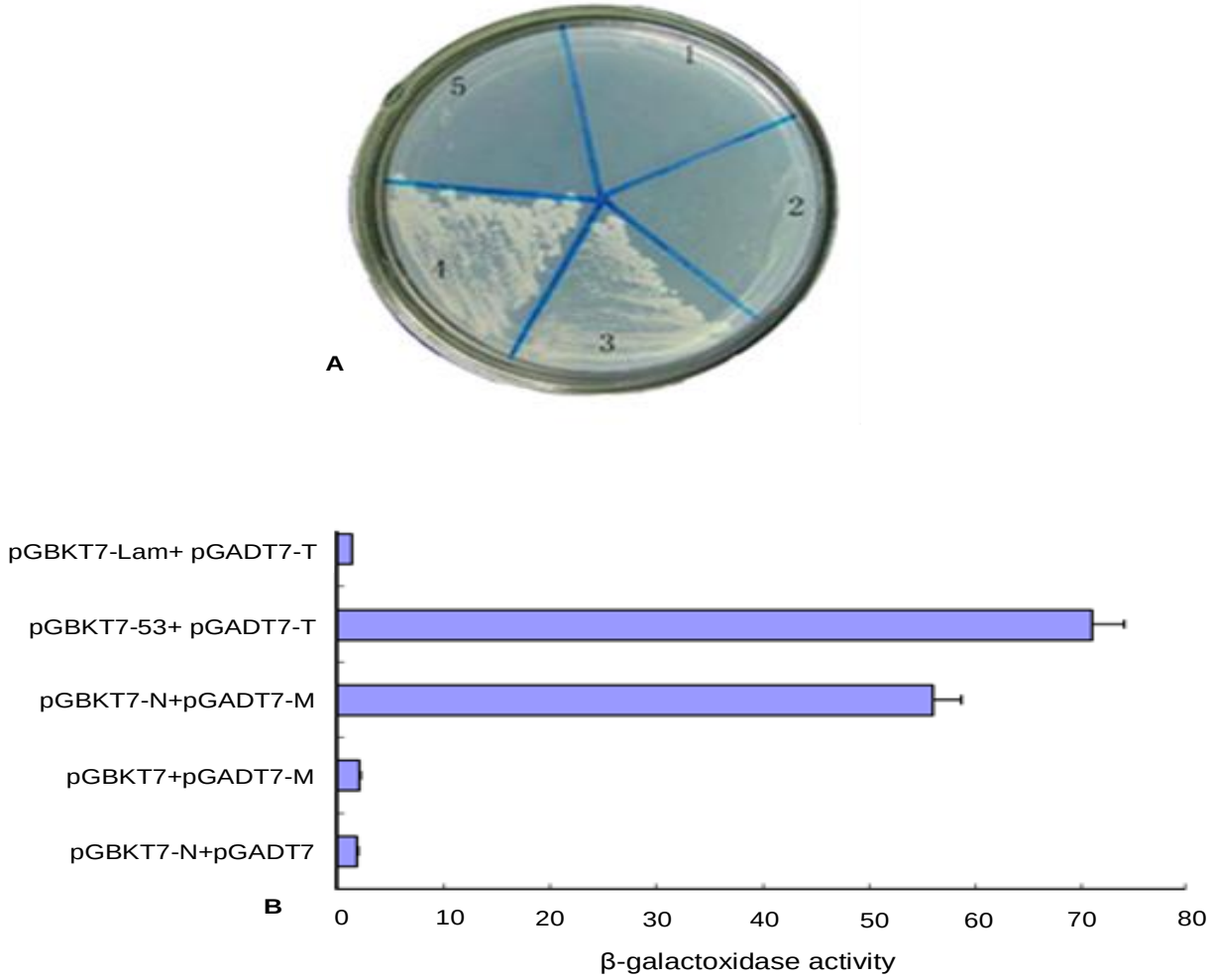

Figure 1. Yeast two-hybrid analysis of the IBV N and M protein interaction. A: Yeast two-hybrid assay of pGBKT7-N+pGADT7; pGBKT7+pGADT7-M; pGBKT7-N+pGADT7-M; positive control, pGBKT7-53+ pGADT7-T; negative control, pGBKT7-Lam+ pGADT7-T; B: Yeast two-hybrid quantification of β -galactosidase activity. The plasmid pairs above were cotransfected into the AH109 yeast strain, and assessed for β-galactosidase activity. Results shown are the average units for triplicate assays. Error bars represent standard deviation.

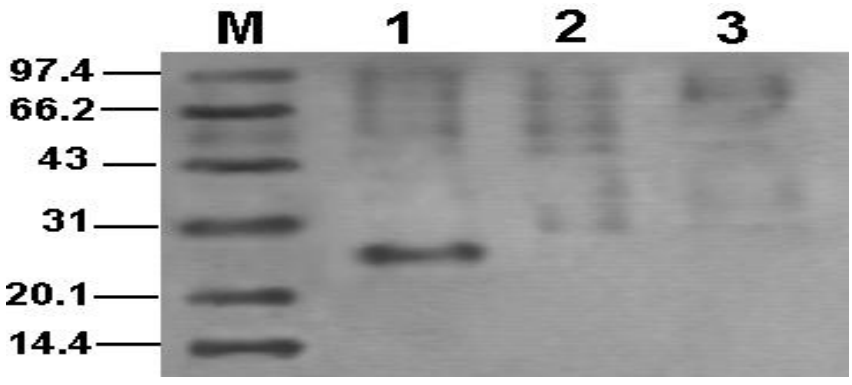

Figure 2. M protein immunoprecipitated with the IBV- N protein. Indicated plasmids were simultaneously transfected into vero cells. Twenty-four hours after transfection, coimmunoprecipitation was performed using anti-HA magnetic microbeads. The proteins immunoprecipitated (IP) were assayed with an anti-myc monoclonal antibody. M, Marker; 1, HA-M+myc-N; 2, HA-M; 3, myc-N.

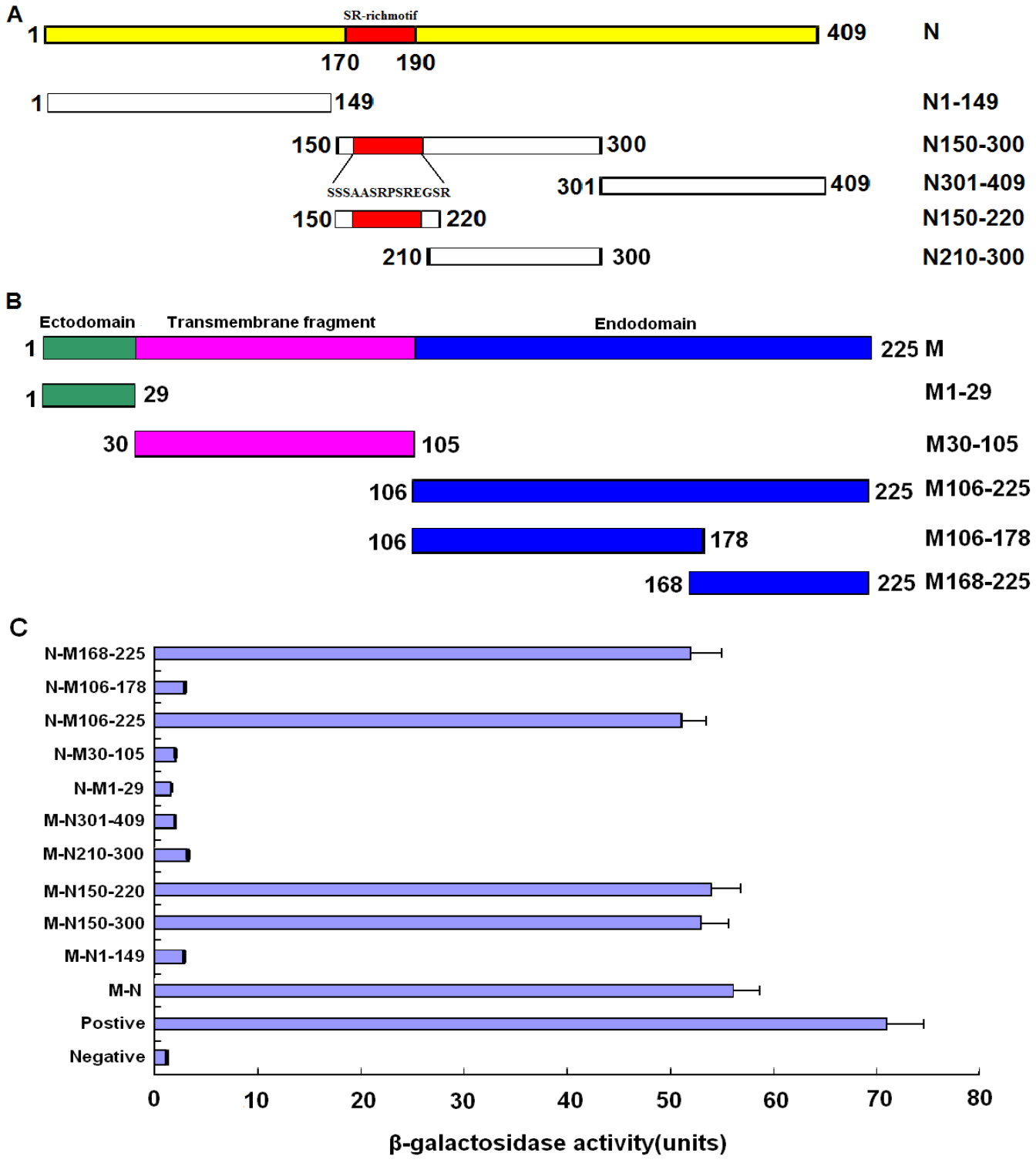

Figure 3. Mapping the domains involved in IBV M/N interaction by the yeast two-hybrid assay. A: Schematic representation of the IBV N protein and the truncated mutants used in the yeast two-hybrid system, the SR-rich motif is highlighted; B: schematic representation of the IBV M protein and the truncated mutants used in the yeast two-hybrid system. M protein is composed of three domains: a short N-terminal ectodomain, a triple-span transmembrane domain and a C-terminal endodomain; the ectodomine, transmembrane fragment and endomain; C: mapping the domains involved in IBV M/N interaction. 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 positive 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.

nucleocapsid with its carboxyl terminus in mouse hepatitis virus (Kuo and Masters, 2002; Narayanan et al., 2000; Kelley et al., 2005), transmissible gastroenteritis corona-virus (TGEV) and severe acute respiratory syndrome (SARS) (Hatakeyama et al., 2008; Hsieh et al., 2008). These results indicated that the C-terminal polar tail within the endodomain of M protein could interact with N proteins, indicating that the large ME probably plays a major role in IBV assembly.

Mapping studies localized the critical N sequences for this interaction to amino acids 150 - 210, which includes the SR-rich motif (containing rich serine and arginine). SR-related proteins are often involved in protein-RNA and protein–protein interactions (Blencowe et al., 1999) and the SR-rich motif is conserved in the N protein of coronavirus. It has been reported recently that SR-rich motif is indispensable for SARS N oligomerization and for N protein interaction with SARS-CoV membrane protein (He et al., 2004). The SR-rich motif is also responsible for the interaction with hnRNPA1 in MHV (Wang and Zhang, 1999) and SARS (Haibin et al., 2005). In addition, this motif is also involved in interaction with SARS Hubc9 (Fan et al., 2006). All these facts indicate that the SR-rich motif of the N protein might play a crucial role in IBV infection. However, this result seems not to be compatible with the reported result that the N protein binds to M protein with its carboxyl terminus in mouse hepatitis virus (Kuo and Masters, 2002) and SARS-CoV (Hatakeyama et al., 2008; Hurst et al., 2009), which may be that IBV nucleocapsid protein possess low homology with others in the coronaviral family.

Our present study revealed that the M-N protein-protein interaction exists in IBV as it does in other members of the Coronaviridae family, thereby reinforcing a notion that the importance of M–N interaction in viral interaction appears to be an essential process for coronaviral assembly (He et al., 2004; Hiscox et al., 2001).

In summary, our data has shown for the first time that the IBV N and M proteins can interact with each other *in vivo* and this interaction appears to be significant for one of the critical steps of viral assembly and maturation process. The SR-rich motif of the N protein and endo-domain of the M protein play crucial role in CoV virus assembly. This current research contributes useful data that will shed light on the molecular mechanism of IBV M/N interaction and provide valuable clues for mutagenic studies in disrupting virion assembly and developing antiviral agents.

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