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Engagement of new castle disease virus (NDV) matrix (M) protein with charged multivesicular body protein (CHMP) 4 facilitates viral replication☆

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ABSTRACT

Newcastle disease virus (NDV) causes heavy economic losses to poultry industry across the globe every year. Although NDV matrix (M) protein is involved in virus budding and our previous data indicate that *in ovo* expression of M protein facilitates NDV replication, the underlying mechanism for the role of M protein in NDV replication is not clear. Using yeast two-hybrid system and immunoprecipitation approaches, we found that M protein interacted with host vacuolar sorting protein charged multivesicular body protein (CHMP) 4B and 4C. In addition, the colocalization of M protein and CHMP4B/C could be observed using a laser confocal scanning microscope. Knockdown of CHMP4B by siRNA or transient expression of CHMP4B/C dominant negative forms markedly inhibited NDV growth in DF-1 cells. Thus, cellular CHMP4s play a critical role in NDV replication.

1. Introduction

NDV is one of the major pathogens affecting poultry industry across the globe. It is a member of the genus *Avulavirus*, the family *Paramyxoviridae* (Seal et al., 2000). The NDV genome contains 15,186 nucleotides with six open reading frames, encoding six structural proteins including nucleocapsid protein, phosphoprotein polymerase protein, M protein and two transmembrane glycoproteins hemagglutinin–neuraminidase and fusion proteins (Faaberg and Peeples, 1988; Seal et al., 2000). Of all these structural components, the M proteins of NDV are considered to be relatively conserved among isolates of different paramyxovirus(Zanetti et al., 2003; Seal et al., 2000; Seal, 1995). M protein may play a pivotal role in virion assembly and release as demonstrated by previous findings that NDV M protein is necessary and sufficient for virus-like particles (VLPs) budding and release in vitro (Pantua et al., 2006; Takimoto and Portner, 2004). Our data also showed that *in ovo* expression of M protein facilitated NDV replication (Wang et al., 2009). In addition, M protein in other viruses has been proposed to interact with fusion protein and/or hemagglutinin–neuraminidase (Ali and Nayak, 2000; Sanderson et al., 1994) and nucleocapsid protein (Stricker et al., 1994), and it inhibits protein synthesis of host cells (Peeples et al., 1992). However, the underlying mechanism for the role of M protein in NDV replication is not clear.

In this paper, we report that the M protein interacts with the two isoforms of Charged Multivesicular Body Protein 4 (CHMP4), the components of the Endosomal Sorting Complex Required for Transport (ESCRT) system. Knockdown of CHMP4B by siRNA or transient expression of CHMP4s dominant negative forms markedly inhibited NDV growth in host cells, suggesting that the engagement of M protein with cellular CHMP4s may be exploited by NDV for its replication.

2. Materials and methods

2.1. Cells and virus

Both DF-1 (immortal chicken embryo fibroblast) and HEK293T cells were obtained from ATCC. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 incubator. F48E9, a velogenic strain of NDV was kindly provided by Dr. Jinhua Liu (China Agricultural University, Beijing, China).

Abbreviations: NDV, Newcastle disease virus; CPE, cytopathic effect; RNAi, RNA interference; CHMP4, charged multivesicular body protein 4; ESCRT, endosomal sorting complex required for transport; ALIX, ALG-2-interacting protein X.

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2.2. Reagents

All the restriction enzymes were purchased from NEB (USA). pRK5-flag, pDsRed-monomer-N1 and pEGFP-C1 vectors were obtained from Clontech (USA). Anti-FLAG (F1804) antibody was purchased from Sigma (USA). Anti-green fluorescent protein (anti-GFP; sc-9996), and anti-β-actin (sc-1616-R) monoclonal antibodies were obtained from Santa Cruz Biotechnology (USA). Rabbit anti-CHMP4B polyclonal antibody (ab105767) was purchased from Abcam (United Kingdom).

2.3. Construction of plasmids

NDV M was cloned from NDV strain F48E9 using specific primers (sense primer 5′-ATGGACTCATCTAGGACAATT-3′ and antisense primer 5′-TTATTTCTTAAAAGGATTGTAT-3′ [GenBank ID: AF089819]). Chicken CHMP4B, CHMP4C and ALIX was cloned from cDNA of DF-1 cells using the following specific primers: for CHMP4B, sense primer 5′-ATGTCGGGGATCCTGGGGAAG-3′ and antisense primer 5′-CATGTTTCCTGCCCAAGCTTC-3′ (GenBank ID: NM001006286); for CHMP4C, sense primer 5′-ATGAGCAGGATCTCCAGGTTCTTCA-3′ and antisense primer 5′-GGAAGCCCAGGCTGCTAACTGCTTC-3′ (GenBank ID: NM001199475); for ALIX, sense primer 5′-ATGACGAACTTCATCTCGGTG-3′ and antisense primer 5′-TTATTGCTGTGGGAAATAAGG-3′ (GenBank ID: NM001030993). pRK5-FLAG-M, pRK5-FLAG-ALIX, pEGFP-M, pEGFP-CHMP4B, pEGFP-CHMP4C, pDsRed-CHMP4B, and pDsRed-CHMP4C expression plasmids were constructed by standard molecular biology techniques. All the primers were synthesized by Sangon Biotech Company (Shanghai, China).

2.4. Yeast two-hybrid screening and colony-lift filter assay

The pGBKT7-M plasmid expressing the fusion protein GAL4-BD-M was used as bait in the yeast two-hybrid screening. The screening was performed per the manufacturer's protocol (Matchmaker Two-Hybrid System 3) and as previously described (Chen et al., 2011). In β-Gal colony-lift filter assay, the prey plasmids expressing the interacting prey proteins were rescued and co-transformed into *Saccharomyces cerevisiae* AH109 with the bait pGBKT7-M plasmids. Positive clones were selected on SD/-Ade/-His/-Leu/-Trp medium and tested for β-galactosidase activity (LacZ+). The pGBKT7-53 and pGADT7-T co-transformed group was used as a positive control. The pGBKT7-53 and pGADT7-T encode a fusion of the GAL4 DNA-BD and AD and murine p53 and SV40 large T-antigen, respectively. Yeast co-transformed with the β-galactosidase positive plasmids turned blue within 20–30 min. The pGBKT7-Lam and pGADT7-T co-transformed group was used as a negative control. The pGBKT7-Lam and pGADT7-T encode a fusion of the GAL4 DNA-BD and AD and Lam and SV40 large T-antigen, respectively. Yeast co-transformed with the β-galactosidase negative control plasmids did not turn blue. Yeast colonies co-transformed with the pGADT7-derivative plasmids and pGBKT7-M plasmids were checked periodically for the appearance of blue colonies, typically from 30 min to 8 h.

2.5. Coimmunoprecipitation and Western blot analysis

For immunoprecipitation, HEK293T cells or DF-1 cells (6×10^5) were seeded on 6-well plates and cultured for 24 h before co-transfected with pRK5-FLAG-M and pEGFP-CHMP4B or pEGFP-CHMP4C or empty vectors as controls by using standard calcium phosphate precipitation. Twenty-four hours after transfection, cell lysates were prepared using a lysis buffer (1% NP-40; 50 mM Tris–HCl, PH8.0; 150 mM NaCl; 5 mM EDTA; 10% glycerol; 10 mM DTT; 1× complete cocktail protease inhibitor). The cell lysates were incubated with 2 μg of anti-FLAG antibody at 4 °C for 2 h, and then mixed with 20 μL of 50% slurry of protein A/G plus-agarose and incubated for another 2 h. Beads were washed three times with the lysis buffer and boiled with 2× SDS loading buffer for 10 min. The samples were fractionated by electrophoresis on a 12% SDS-PAGE gels and resolved proteins were transferred onto a PVDF membranes. After blocking with 5% skim milk, the membranes were incubated with either anti-GFP or anti-FLAG antibodies followed by an appropriate HRP-conjugated secondary antibody. Blots were developed using an enhanced chemiluminescence (ECL) kit. For endogenous CHMP4B pull-down assay, DF-1 cells were transfected with pRK5-FLAG-M or with empty vector. Twenty-four hours after transfection, the cell lysates were subjected to immunoprecipitation with anti-FLAG antibody and immunoblotted with anti-CHMP4B or anti-FLAG antibodies.

2.6. Confocal laser scanning microscopy assay

DF-1 cells (1×10^5) were seeded on coverslips in 24-well plates and were cultured overnight before transfection with pEGFP-M and pDsRed -CHMP4 C. Twenty-four hours after transfection, the cells were fixed with 1% paraformaldehyde and the nuclei were stained with DAPI.

2.7. RNA isolation and qRT-PCR analysis

Total RNA was prepared from DF-1 cells using Qiagen RNeasy kit per manufacturer's instruction. One microgram of total RNA was used for cDNA synthesis by reverse transcription. For supernatant samples preparation, 5 μL extracted RNA was used for cDNA synthesis by reverse transcription. Both reverse transcriptions were using RT-PCR kit (TaKaRa). The specific primers for NDV M (5′-AGTGATGTGCTTGGACCCTC-3′, 5′-CCTGAGGAGAGGCATTTGCTA-3′) and GAPDH (5′-TGCCATCACAGCCACACAGAAG-3′, 5′-ACTTTCCCCACAGCCTTAGCAG-3′) were designed with reference to previous publications (Wise et al., 2004; Heidari et al., 2008) and synthesized by Sangon Company (Shanghai, China). The real-time PCR was carried out using the Light Cycler 480 (Roche, Switzerland). The PCR was performed in a total volume of 20 μL containing 1 μL cDNA, 10 μL 2× SYBR Green Premix Ex Taq (TaKaRa), 0.4 μM of each gene-specific primer. Thermal cycling parameters were as follows: 94 °C for 2 min, 40 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s, and followed by one cycle of 95 °C for 30 s, 60 °C for 30 s, and 95 for 30 s. The final step was to obtain a melt curve for the PCR products to determine the specificity of the amplification. For cell pellet sample analysis, GAPDH was utilized as the reference gene and expression levels of NDV M gene were calculated relatively to the expression of the GAPDH. The relative fold change of M gene expression in the cell pellets was calculated as follows: (mRNA expressions of M gene/GAPDH in Control RNAi or Normal cells)/(mRNA expressions of M gene/GAPDH in CHMP4B RNAi cells). The relative fold change of m gene expression in the supernatant was calculated as follows: (mRNA expressions of M gene in Control RNAi or Normal cell cultures)/(mRNA expressions of M gene in CHMP4B RNAi cell culture).

2.8. RNAi knockdown of CHMP4B

siRNAs designed by a Genechem Company (Shanghai, China) were used to knockdown CHMP4B in DF-1 cells. The sequences of siRNAs for targeting CHMP4B in DF-1 cells included:

RNAi#1 (sense, 5′-GGACAUUGCGGAGCAGCAAtt-3′; antisense, 5′-UUGCUGCUCCGCAAUGUCCtt-3′), RNAi#2 (sense, 5′-GGAAUUUGAUGAGGAUGAAtt-3′; antisense, 5′-UUCAUCCUCAUCAAAUUCCtt-3′), RNAi#3 (sense, 5′-GGAUGAACUCAUGGCAGAAtt-3′;