



Domain I and II from newly emerging goose tembusu virus envelope protein functions as a dominant-negative inhibitor of virus infectivity

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ABSTRACT

Flavivirus envelope protein locates at the outermost surface of viral particle and mediates virus entry and fusion infection, and domains I and II of E protein play an important role in this process. In this study, we have expressed and purified goose tembusu virus (GTV) E protein domains I and II (DI/II) from *E. coli*, and tested conceptual approach that purified protein serves as anti-viral reagent. We found that DI/II inhibited GTV JS804 infection in BHK-21 cells in a dose-dependent manner, and this inhibition activity was achieved by binding to cell membrane specifically. Moreover, JS804 treated with DI/II specific antiserum decreased its infectivity to BHK-21 cells. Taken together, this is first to show that the purified DI/II domain of tembusu virus expressed in *E. coli* was able to interfere with virus infection, which opens an avenue to develop novel anti-viral regents to prevent and eventually eradicate GTV infection.

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1. Introduction

In April 2010, a severe disease spread out in most duck- and goose-farming regions in China including Zhejiang, Jiangsu, and Fujian provinces. The disease continued to spread until winter, when the shelducks and geese in the provinces of Shandong, Henan, Hunan, Hubei, and Jiangxi were under a huge threat by this pathogen (Huang et al., 2013). Affected egg-laying ducks showed a reduction in egg production of 20–90% (Wang et al., 2011). Other clinical signs included acute anorexia, antisocial behavior, rhinorrhea, diarrhea, ataxia, and paralysis, with a morbidity of 100% and mortality of 5–30%, possibly due to secondary bacterial infections (Yan et al., 2011). Recent studies indicated that domestic chickens were susceptible to this emerging disease with similar clinical signs (Cao et al., 2011; Liu et al., 2012a, 2012b; Su et al., 2011; Tang et al., 2012). The new disease has become one of the most economically important infectious diseases of ducks in China (Wang et al., 2011).

Laboratory and field investigations determined that the goose egg drop syndrome was caused by a new flavivirus, named JS804 virus, closely related to Tembusu virus (Huang et al., 2013).

Flaviviruses are single-stranded positive-sense RNA viruses classified in the Genus *Flavivirus* (Han et al., 2013; Hoshino et al., 2009),

the *Flavivirus* genus (family *Flaviviridae*) consists of nearly 70 viruses. Many, such as Japanese encephalitis virus, yellow fever virus, West Nile virus, and tick-borne encephalitis virus can lead to signs from mild febrile disease, encephalitis, hemorrhagic fever, and shock syndrome to death in both humans and animals (Brault et al., 2011). The flavivirus genome, is approximately 10.5 kb which contains one single open reading frame (ORF) and encodes a polyprotein (Zou et al., 2009). The polyprotein is then cleaved into three structural proteins (capsid [C]; membrane [M], and envelope [E]) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) by viral and cellular protease, and these nonstructural proteins are responsible for virus replication, proteolysis, and maturation (Lindenbach and Rice, 2003; Mukhopadhyay et al., 2005).

The major envelope protein (E) of flavivirus is a receptor binding protein, which is the primary determinant of host range, cell tropism and virulence and a major antigen in eliciting neutralizing antibodies during the immune response (Chu et al., 2005; Roehrig, 2003). In addition, E protein mediates virus and host membrane fusion in the acidic milieu of the late endosomes (Chu and Ng, 2004; Heinz and Allison, 2003). E protein consists of three domains: a structurally central amino terminal domain (domain I, DI), a dimerization domain (domain II, DII) and a carboxyl terminal Ig-like domain (domain III, DIII) (Rey et al., 1995). As a central unit of E protein complex, DI adopts β -barrel structure and acts as a bridge-like hinge linking the extended DII and the globular DIII together. Histidines in the hinge region are important triggers for conformation change of DII and DIII upon pH change in the processes of viral and cell membrane fusion and homodimer formation/dissociation. DII is composed of two segments that project from loops of DI. The large

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segment of DII, which is composed of twisted β strands, is stabilized by disulfide bonds and contains a highly conserved hydrophobic fusion loop at its tip, known as the fusion peptide (FP). The FP is responsible for the direct interaction with target membrane when fusogenic conformation is achieved (Mendes et al., 2012; Yu et al., 2013). DIII is located at the C-terminus of E protein and contains several epitopes which can elicit virus-neutralizing antibodies (Chu et al., 2005). In addition, other studies have shown that DIII mediated flavivirus attachment to host cells (Anderson, 2003).

Since the domains I and II play important roles in flavivirus entry, they are attractive targets to block infectivity. In the present study, domains I and II of goose tembusu virus were cloned, expressed, refolded and purified. The recombinant protein DI/II was tested for competitive inhibition of goose tembusu virus entry.

2. Materials and methods

2.1. Cells, viruses and antibodies

BHK-21 cells were maintained in DMEM containing 10% inactivated fetal calf serum (FCS) plus 100 μ g/ml of penicillin/streptomycin. All cells were grown at 37 °C in an atmosphere of 5% CO₂. Goose tembusu virus (GTV) JS804 was isolated from an affected goose with neurological clinical signs, propagated in BHK-21 cells and used in this study. When 75% of the cells in culture demonstrated CPE (the cells rounding up and floating free from the surface of the flask), the cultures were collected as stock virus and stored at –70 °C for further use. Serial 10-fold dilutions of the stock virus were used to determine the 50% Tissue Culture Infective Dose (TCID₅₀). The dilutions were inoculated into a monolayer of BHK-21. The virus was allowed to attach to the cell monolayer for 2 h at 37 °C and then removed prior to being covered with a mixture of 1% agarose and cell culture media. The cells were observed daily for plaque production for four days, and TCID₅₀ was calculated according to the Reed–Muench's method (Reed and Muench, 1938). Monoclonal antibody against JS804 E protein was produced by this lab (Niu et al., 2013). Anti-FLAG tag monoclonal antibody and alkaline phosphatase-conjugated goat anti-mouse IgG were purchased from the Beyotime Institute of Biotechnology.

DIII or entire E gene was cloned into pET28a vector in frame with FLAG. The resulting plasmids were transformed, and the respective proteins were expressed in *E. coli* [strain BL21(DE3)] and purified by Anti-FLAG M2 Affinity Gel according to the manufacturer instructions (Sigma).

2.2. Cloning and expression of recombinant GTV DI/II protein

Gene fragment of N-terminal 1–298 aa (DI and DII) of JS804 E protein was amplified by PCR using following primers. Forward primer, 5'-GAATTCATGGACTACAAAGACGACGACGACAAATTCAGCTGCTGGGGATGC-3' and reverse primer, 5'-GTGACTCATTTGTCGTCGTCGTCTTTGTAGTCTTTCAGCTTCAAACCTGC-3'. The *Eco*R I and *Sal* I restriction sites are underlined. The sequence of FLAG tag (DYKDDDDK) is italicized. The PCR product of 894 bp was digested with the restriction enzymes *Eco*R I and *Sal* I and ligated into pET28a vector digested with the same restriction enzymes. The resulting construct was designated as pET-28a-DI/II and confirmed by DNA sequencing. BL21(DE3) transformed with pET-28-DI/II were grown in LB with shaking at 37 °C until OD₆₀₀ of the culture reached 0.4–0.6, and IPTG (isopropyl- β -D-thiogalactoside) was added to a final concentration of 1 mM for additional 4–6 h. Cells were harvested by centrifugation (14,000 g for 5 min) and washed three times with PBS buffer. Cells were resuspended in PBS buffer and lysed by ultrasonic cell disruptor followed by centrifugation (14,000 g for 30 min at 4 °C). The protein expression was confirmed by running

the crude lysates on SDS-polyacrylamide gel and visualized by Coomassie staining.

2.3. Purification of recombinant GTV DI/II protein

Inclusion bodies were solubilized in 8 M urea and the proteins were purified by anti-FLAG M2 Affinity Gel following the instructions of the Sigma Company. The purity of purified protein was examined by SDS-PAGE and confirmed by Western blot with anti-FLAG antibody (Beyotime) and anti-E monoclonal antibody as described above.

2.4. Generation of murine polyclonal antibodies against GTV DI/II protein

Seventy micrograms of DI/II was mixed with an equal volume of Freund's complete adjuvant (Sigma). The antigen–adjuvant mixture was applied to 6-week-old female BALB/c mice three times subcutaneously at 14-day intervals. Mouse sera were collected 12 days after the last booster and stored at –20 °C. The titer of polyclonal antiserum was assessed by indirect enzyme linked immunoassay (ELISA) established with purified protein DI/II as capture antigen and HRP-conjugated goat-anti-mouse IgG was used as detection antibody, respectively. Different dilutions of polyclonal antiserum (1:50–1:51,200) were used to measure the titer of polyclonal antiserum by indirect ELISA.

2.5. Inhibition of goose tembusu infection by GTV DI/II protein

Different concentrations of DI/II, BSA, DIII, or E proteins were added into BHK-21 cells (1×10^6 cells) and incubated at 4 °C for 1 h. Unbound proteins were removed by washing the cells three times with PBS. The cells were then infected with 200 TCID₅₀ GTV JS804 for 2 h at 37 °C. Unbound virus was removed by washing three times with PBS. Total RNAs were extracted from infected cells (AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit, AXYGENT), and the virus nucleic acid was detected by reverse transcriptase real-time PCR assay to examine virus entry. The primers used for real-time PCR were: EF primer (forward, 5'-GTGAGATCTTACTGCTATGAG-3') and the ER primer (reverse, 5'-ACTTGGCACATGTCTGTATGC-3'). Three independent experiments were carried out.

2.6. DI/II and cell membrane binding assay

BHK-21 cells grown on glass coverslip in 12-well plate were incubated with 50 μ g/ml of purified protein DI/II, DIII, E or BSA at 4 °C for 1 h. Unbound proteins were removed by washing cells three times with PBS. The cells were fixed with 4% paraformaldehyde permeabilized with 0.1% Triton X-100, and blocked with 5% BSA in PBS, the cells were then incubated with a mouse anti-FLAG antibody (Beyotime) followed by incubation with FITC conjugated goat anti-mouse IgG antibody. The cells were then examined by fluorescence microscope.

2.7. Plaque neutralization assay

To evaluate the neutralization ability of antiserum of mice immunized with protein DI/II, virus plaque reduction assay was performed. Antiserum was diluted in serial twofold in DMEM, each dilution (from 1:2 to 1:2048) was mixed with 200 TCID₅₀ of JS804 virus and incubated at 37 °C for 1 h and the residual infectivity of JS804 virus was determined by plaque assay as described above.

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