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Research in Veterinary Science 98 (2015) 121–126

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

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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ARTICLE INFO

Article history:
Received 13 June 2014
Accepted 6 November 2014

Keywords:
Goose tembusu virus
Envelope protein
Domain I and II
Inhibition

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.

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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http://dx.doi.org/10.1016/j.rvsc.2014.11.003
0034-5288/

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 μg/ml of penicillin/streptomycin. All cells were grown at 37 °C in an atmosphere of 5% CO_2. 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 ($TCID_{50}$). 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 $TCID_{50}$ 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'-GAATTCATG*GACTACAAAGACGACGACGACAAA*TTCAGCTGT CTGGGGATGC-3' and reverse primer, 5'-GTCGACTCA*TTTGTCGTCGTCG TCTTTGTAGTC*TTTCAGCTTCAAACCCTGC-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 OD_{600} of the culture reached 0.4–0.6, and IPTG (isopropy-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 $TCID_{50}$ 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, AXYGEN), 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 μg/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 $TCID_{50}$ 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.

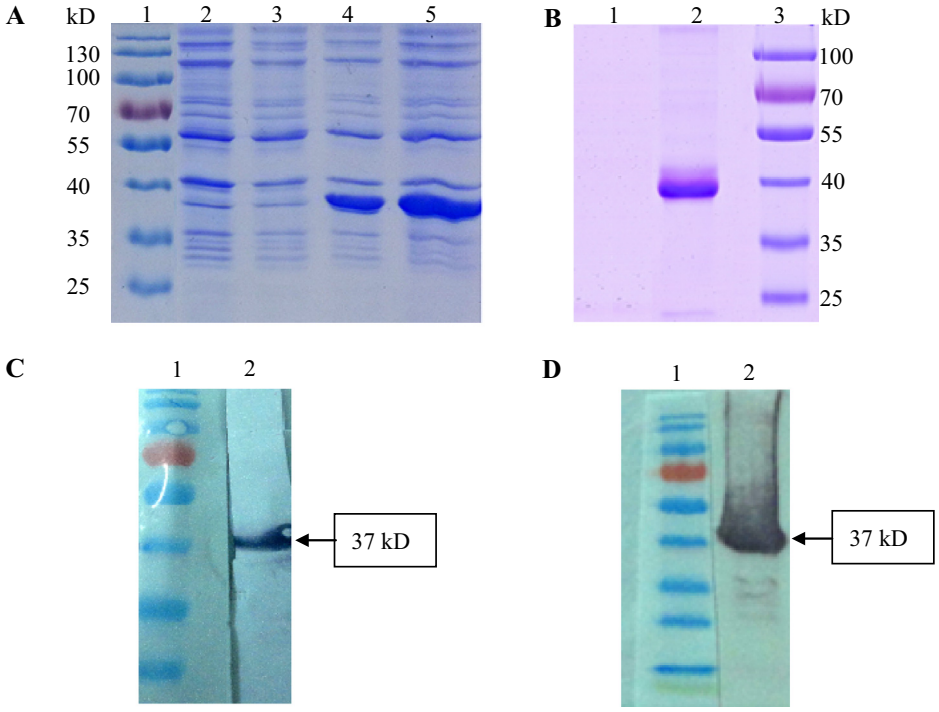

Fig. 1. Expression of recombinant GTV E DI/II protein and detection by western blot. (A) Induction of DI/II from *E. coli*. Lane 1, molecular weight marker; lane 2, crude lysates from *E. coli* (BL21) with pET-28a vector; lane 3–5, crude lysates protein from *E. coli* (BL21) with pET-DI/II after IPTG induction for 0, 4, 6 h, respectively. (B) Detection of E DI/II protein after purification. Lane 1, soluble supernatant after cell sonication; lane 2, pellet fraction after cell sonication; lane 3, molecular weight marker. (C) Detection of purified GTV E DI/II with anti-E monoclonal antibody. Lane 1, molecular weight marker; lane 2, the recombinant E DI/II protein is recognized by the anti-E monoclonal antibody. (D) Detection of purified GTV E DI/II with anti-FLAG antibody. Lane 1, molecular weight marker; lane 2, the recombinant E DI/II protein is recognized by the anti-FLAG antibody.

2.8. Cytoxicity assay

To determine whether protein DI/II is toxic to BHK-21 cell, the cell mitochondrial reductase activity was measured after addition of protein with Cell Counting Kit-8 (CCK-8, Dojindo) according to the manufacturer's protocol. Briefly, 100 μg/ml of DI/II, DIII, E or BSA in serum-free DMEM were added to BHK-21 monolayers in 96-well plates for 1 h at 4 °C and then incubated at 37 °C with 5% CO_2 for 44 h. Then CCK-8 was added to each well and incubated at 37 °C with 5% CO_2 for 4 h. Absorbance at 450 nm was measured using a BioTek microplate reader.

3. Results

3.1. Expression and purification of the recombinant GTV E domain I and II protein

We have cloned N-terminal part (amino acids 1–298) of envelope E protein of JS408 in frame with Flag tag in pET28a expression vector; this part of E contains domain I and II. After transformation of this construct into *E. coli* BL21 strain and induced by addition of 1 mM IPTG, a 37 kDa protein was readily induced which was apparently visualized from crude cell lysates separated on SDS-PAGE gel followed with Coomassie staining (Fig. 1A, lanes 5 and 6), and a near identical protein with similar molecular weight was purified by affinity purification with anti-Flag tag antibodies (Fig. 1B). More importantly, the purified protein was recognized by anti-E monoclonal antibody and anti-FLAG antibody as confirmed by immunoblotting assay (Fig. 1C and D).

3.2. Competitive inhibition of virus entry with recombinant GTV DI/II protein

Domains I and II of envelope protein E of GTV have been proposed to have an important role in virus entry. To test if truncated E protein that contains domains I and II competes with endogenous E in mediating virus entry, we performed virus entry assay and examined viral RNA level inside of cells by real-time PCR as described in Section 2, Material and methods. As shown in Fig. 2, viral RNA level was significantly lower in DI/II treated cells compared with

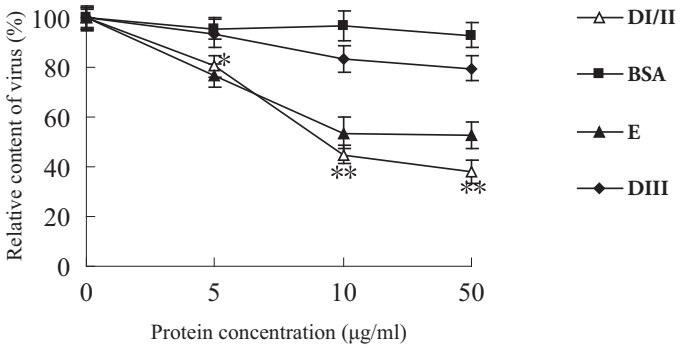

Fig. 2. Inhibition of GTV entry by DI/II protein. BHK-21 cells were first incubated with different concentrations of proteins. GTV was added and assayed for virus entry by reverse transcriptase real-time PCR assay. Data were presented from three independent experiments and statistic analysis was done with SPSS software. * Denotes statistically significant difference and ** denotes statistically extremely significance.

BSA, or DIII treated cells, suggesting that virus entry into cell was inhibited by protein DI/II in a dose-dependent manner. Pretreatment with a concentration of 50 μg/ml of recombinant DI/II protein resulted in 60% inhibition in GTV entry.

3.3. Recombinant GTV DI/II protein binds to the surface of BHK-21 cells

Given that DI/II inhibited JS804 entry into cells, we set to investigate the mechanism of inhibition. We wanted to know if DI/II binds to cell membrane and this binding blocks JS804 E protein access to cell membrane. We added purified DI/II into BHK-21 monolayer and incubated the cells for 1 h at 4 °C, and washed out the excess protein with PBS followed with indirect immunofluorescence assay with specific antibody to examine whether DI/II was detectable. As shown in Fig. 3, specific fluorescence on cell membrane was observed from DI/II treated cells (panel C), but not from BSA- or DIII-treated cells (panel A and B). These results suggest that the N-terminal part of E protein which contains domains I and II is sufficient to bind to cell membrane.

3.4. Murine polyclonal antibodies to recombinant DI/II protein neutralized GTV

To further verify the specificity of DI/II blocking virus entry, we performed plaque neutralization assay by using anti-DI/II specific serum. Murine polyclonal antibodies against DI/II were produced and the titers of the antiserum were assessed by indirect ELISA that was established with purified DI/II as capture antigen (data not shown). Plaque neutralization assays were then carried out as described in material and methods. As shown in Fig. 4, virus titer in lower dilution (1:2–1:64 dilution) antiserum-treated cells decreased 90% compared with that of preimmune serum-treated cells. We concluded that DI/II specific antiserum neutralized JS804 virus.

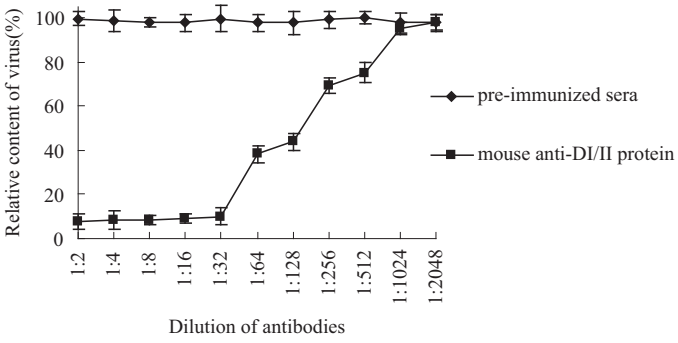

Fig. 4. Plaque neutralization of GTV JS804 with murine polyclonal antibodies against DI/II protein. Antiserum was diluted in serial twofold in DMEM, each dilution (from 1:2 to 1:2048) was mixed with 200 $TCID_{50}$ of JS804 virus and incubated at 37 °C for 1 h and the residual infectivity of JS804 virus was determined by plaque assay. Data were presented from three independent experiments and statistic analysis was done with SPSS software.

More than 50% neutralization of the virus was maintained until the murine polyclonal antibodies were diluted to 1:128. At greater dilutions, there was an exponential decrease in the degree of neutralization. However, when the dilution factor was 1:1024, there was only about 10% neutralization of the virus. From these results, we concluded that DI/II specific antiserum neutralized JS804 infectivity.

3.5. Cytoxicity assay

In order to make sure that the inhibition of recombinant DI/II protein is not due to underlying cytoxicity, we tested whether purified proteins DI/II are toxic to BHK-21 cells. BHK-21 monolayers were exposed to 100 μg/ml concentrations of DI/II for 48 h, cell

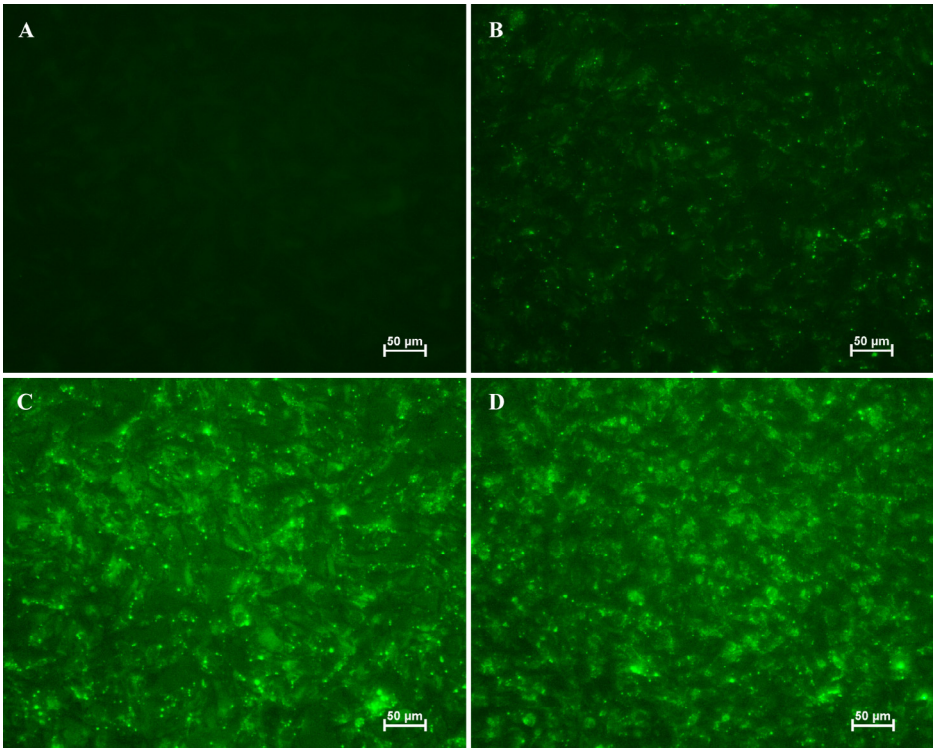

Fig. 3. DI/II binds to the membrane of BHK-21 cells. BHK-21 cells were incubated with BSA (A), DIII (B), recombinant DI/II protein (C) and entire E protein (D) at 4 °C for 1 h and then washed repeatedly by PBS. The bound proteins were detected by anti-E monclonal antibody and visualized with fluorescence microscopy. Bars = 50 μm.

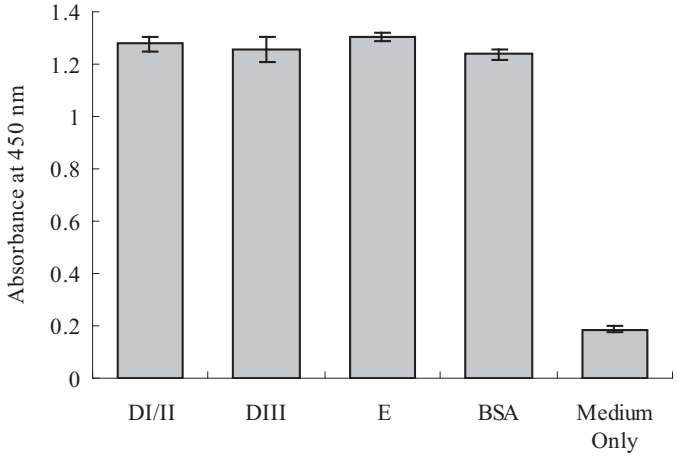

Fig. 5. Cytotoxic assay. CCK-8 assays for cell viability were performed after 48 h incubation with 100 μg/ml of DI/II, DIII, E and BSA. The medium without cells was taken as blank control. Data were presented from three independent experiments and statistic analysis was done with SPSS software.

viability was evaluated by measuring the mitochondrial reductase activity. As shown in Fig. 5, there is no significantly difference in viable cell number among all the treatment even in the presence of 100 μg/ml protein, suggesting that DI/II was non-toxic to BHK-21 cells.

4. Discussion

The outbreak and rapid spread of an egg drop syndrome in the major duck/goose-producing regions of China resulted in serious economic loss. Despite inactivated (Li et al., 2013) and live attenuated vaccines have been evaluated in duck models (Li et al., 2014), the risk of antibody dependent enhancement with inactivated virus and safety concerns with live virus vaccine are highly debatable (Chu et al., 2007).

Jiang et al. (1993) demonstrated that a synthetic peptide, corresponding to the sequence (637–666) of the HIV-1 glycoprotein gp41, could block virus infection. Subsequently, additional peptide mimics of the fusion proteins of other retroviruses, orthomyxoviruses, paramyxoviruses, filoviruses, coronaviruses, and herpesviruses have also been identified and shown to inhibit viral entry. Synthetic peptides corresponding to sequences in domain I, II, III and stem of dengue virus and West Nile virus E proteins were identified to inhibit the infectivity of these viruses (Hrobowski et al., 2005).

In this study, we showed for the first time that GTV E truncated protein DI/II is sufficient to inhibit JS804 infection. The inhibitory effects of DI/II were dose dependent in the range of 5–50 μg/ml (Fig. 2). It is of note that the inhibition activity of DI/II is higher than that of E protein ($P > 0.05$). It is possible that E protein with C-terminal truncation binds to cell membrane better.

The first step for flaviviruses to enter cells involves binding of E protein to cell receptor (Smit et al., 2011). Flaviviruses must recognize an ubiquitous cell surface molecule or utilize multiple receptors for cell entry (Rodenhuis-Zybert et al., 2010). E protein was initially postulated to form the receptor binding site for the virus particles because of its exposed location at the surface of the virus (Chu et al., 2005). Crystallographic studies on DENV-DC-SIGN complexes revealed that interaction with DC-SIGN is preferentially mediated through DII (Smit et al., 2011). This study provided direct evidence, for the first time, that DI/II of the GTV E protein is involved in binding to cell surface molecules for BHK-21 cells (Fig. 3). We do not know the exact mechanism of how DI/II inhibits JS804 infection; however, our results suggest that DI/II binds to cell membrane and this binding block virus access to cell receptor, and this is ongoing research we focus.

In a conclusion, our finding that excess DI/II protein inhibits GTV infection provides novel conceptual target for designing anti-viral agent or vaccine to prevent and eventually eradicate tembusu virus infection.

Acknowledgements

The project was supported by the Natural Science Foundation of Jiangsu Province (Grants No. BK2012376) and National Natural Science Foundation of China (No. 31172345).

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