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The assembly of virus-like particles of porcine transmissible gastroenteritis virus in vitro by baculovirus expression system

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Abstract: Virus-like particles (VLPs) produced by recombinant expression of the major viral structural proteins could be an attractive method for transmissible gastroenteritis virus (TGEV) assembly and release. In this study, the *M* gene, *sM* gene, and *N* gene of TGEV were inserted into donor plasmid pFastBac Dual. Those recombinant donor plasmids were transformed into *E.coli* DH10Bac respectively. Recombinant baculoviruses were obtained by transfection of the Sf9 cells with Bacmid-M, Bacmid-sM, and Bacmid-N. The results of indirect immunofluorescence assay showed that recombinant M protein, sM protein, and N protein were expressed successfully. In vitro experiments of VLP assembly of TGEV were performed by infection of insect cells with various combinations of recombinant baculoviruses identified. It is shown that expression of M protein alone, coexpression of M plus sM protein, and coexpression of M plus N protein in Sf9 insect cells infected by recombinant baculovirus could result in the occurrence of a virion morphologically similar to TGEV. However, the sM and N protein could not generate a structure similar to the virion of TGEV when expressed alone. This study provides a theoretical foundation for further study on the role of TGEV structural proteins in the assembly process of virions.

Key words: Transmissible gastroenteritis virus, virus-like particles, baculovirus expression system

1. Introduction

Transmissible gastroenteritis virus (TGEV) is the etiological agent of transmissible gastroenteritis, which is a condition associated with high morbidity in animals of all ages and high mortality in sucking piglets (1). TGEV is a member of the Coronaviridae family, possesses a large 28.5-kb single-stranded sense RNA genome, and comprises four structural proteins encoded by the spike (*S*), membrane (*M*), envelope (*sM*), and nucleoprotein (*N*) genes. The S protein forms the peplomers on the virion envelope and features a major antigenic site (2,3). The M protein is embedded in the lipid envelope, taking part in virus-like particle (VLP) assembly, and the N protein is associated with the genomic RNA to form the nucleocapsid, inducing cell immunity of infected animals (4,5). The small sM protein is localized in the perinuclear region of infected cells and plays an important but not fully understood role in viral morphogenesis and budding. Previous studies on coronaviruses, including TGEV, have demonstrated that the sM protein is essential for coronavirus particle assembly (6). Coronaviruses are assembled intracellularly at membranes of the intermediate compartment, between the endoplasmic reticulum (ER) and the Golgi complex (7–11). The highly basic N interacts with genomic RNA to form helical nucleocapsids (12), and M interacts with nucleocapsids on cell membranes at the ER or Golgi complex (13,14). S and sM are also translated on membrane bound polysomes, inserted into the ER, and transported to the Golgi complex. This complex is where sM and M proteins interact and trigger virion budding (15). S is incorporated into virions via interactions with M. Virions accumulated in vesicles are fused with the plasma membrane and released into the extracellular space eventually (16).

Work on TGEV has established that M protein may serve to initiate the viral particle assembly process through interactions with genomic RNA and nucleoprotein in pre-Golgi compartments. Baudoux et al. found that coexpression of the M and sM proteins led to the formation of VLPs in cells, and pseudoparticles resembling authentic virions were released in the culture medium (17). Here we demonstrate that expression of M protein alone in insect Sf9 cells infected by recombinant baculovirus could form a virion morphologically similar to TGEV. This finding is not in accordance with previous results. Our studies also found that TGEV VLPs could be recovered in insect cells coexpressing a combination of M plus sM or M plus N proteins.

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2. Materials and methods

2.1. Cell and culture conditions

Sf9 cells were maintained at 27 °C in Grace's insect medium supplemented with 10% fetal bovine serum and 10 μg/mL gentamicin.

2.2. Plasmid constructions

The plasmid genes encoding the membrane protein (*M*), the small envelope protein (*sM*), and the nucleocapsid protein (*N*) of TGEV, cloned into a PMD18-T vector (TaKaRa, Japanese), were constructed in our laboratory. The full-length *M*, *sM*, and *N* genes were amplified by polymerase chain reaction (PCR) using primers as follows: *M* gene forward primer 5′-GGGGGAT-CCCCACCATGAAGATTTTGTTAAT-3; *M* gene reverse primer 5′-GGGGAATTCTTATAC-CATATGTAATAATTTTTCTTG-3′; *sM* gene forward primer 5′-GGGCCCGGGATGAC-GTTTCCTAGGGCATTG-3′, *sM* gene reverse primer 5′-GGGGGTACCTCAAGCAAGGAGT-GCTCCATC-3′; *N* gene forward primer 5′-AACCCCGGGATGGCCAACC-3′, *N* gene reverse primer 5′-AGCTCGAGCATCTCGTTTAG -3′. The *M* gene was subcloned into the pFastBac Dual plasmid vector (Invitrogen, USA) between the *BamH I* and *EcoR I* restriction sites, the *sM* gene was subcloned into the pFastBac Dual plasmid vector between the *Sma I* and *Kpn I* restriction sites, and the *N* gene was subcloned into the pFastBac Dual plasmid vector between the *Sma I* and *Xho I* restriction sites. The correct orientation of the insertions was examined by PCR and restriction enzyme analysis. Plasmid pFastBac Dual-M, pFastBac Dual-sM, and pFastBac Dual-N were transformed into *E. coli* DH10Bac respectively. Recombinant baculovirus rBac-M, rBac-sM, and rBac-N were obtained by transfection of the Sf9 cells with the constructs. The recombinant baculoviruses individually expressed the M, sM, and N proteins.

2.3. Immunofluorescence analysis

The Sf9 cells were grown in chamber slides and infected with the recombinant baculovirus at a multiplicity of infection of 5 for 48 h, the supernatant was removed, the cell sheet was fixed in cold acetone for 20 min at –20 °C and stained by an immunofluorescence assay (IFA) technique, and then immunofluorescence analysis was performed as described previously on a Zeiss LSH 510 microscope.

2.4. Transient transfections and production of TGEV VLPs

A total of 8×10^5 cells were incubated in 35-mm plates and transfected with plasmid constructs using Cellfection II Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 8 μL of Cellfection II Reagent was mixed with Grace's medium followed by incubation for 30 min at room temperature, and then 1 μg of plasmid was added. The Cellfection II-plasmid mixture was incubated for 30 min at room temperature and then 210 μL of Cellfection II-plasmid mixture was added dropwise onto the cells. After 5 h of incubation at 27 °C, the transfection mixture was discarded and replaced with 2 mL of complete growth medium (Grace's insect medium, supplemented with 10% FBS). Cells were incubated at 27 °C for 72 h.

In vitro experiments of VLP assembly of TGEV were performed by infecting insect cells with various combinations of identified recombinant baculoviruses (rBac-M, rBac-sM, rBac-N, rBac-M+rBac-sM, rBac-M+rBac-N). The VLPs were observed in insect cells using a transmission electron microscope.

2.5. Purification of TGEV VLPs

At 72 h after transfection, cell lysates was collected and cleared by centrifugation at a low speed (1000 × *g* for 10 min) to pull down cell debris. After passage through a filter with 0.45 μm pore size, cleared cell medium was loaded on top of 20% sucrose cushions and ultracentrifuged for 3 h at 30,000 rpm using a SW41 rotor (Beckman Coulter, Inc., USA). VLP-containing pellets were resuspended in TNE buffer (50 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA, pH 7.4).

2.6. Electron microscopy

Negative staining was performed by standard techniques described previously. Briefly, cell lysates were adsorbed to UV-activated copper grids for 2 min at room temperature. Grids were washed with DB twice and stained with 2% uranyl acetate for 30 s. Samples were visualized in a JEOL 1200 EX II transmission electron microscope.

3. Results

3.1. Amplification and construction of the recombinant bacmid plasmid

The target fragments of the *M*, *sM*, and N genes, with an expected size of 800 bp, 260 bp, and 1200 bp, were amplified from recombinant plasmid templates of PMD18-M, PMD18-sM, and PMD18-N by PCR (Figures 1A–1C). The *M*, *sM*, and *N* PCR products were cut with *BamH I* and *EcoR I*, *Sma I* and *Kpn I*, and *Sma I* and *Xho I*, respectively, and subcloned into the pFastBac Dual vector. The cloning was confirmed by enzyme restriction (Figures 1D–1F). Plasmid pFastBac Dual-M, pFastBac Dual-sM, and pFastBac Dual-N were transformed into *E. coli* DH10Bac, followed by extraction of genome DNA, and expectant fragments were obtained by PCR identification using M13 primers (Figures 1G–1I).

3.2. Identification of M, sM, and N protein expressed in Sf9 cells

The venoms of rBac-M, rBac-sM, and rBac-N were inoculated respectively into Sf9 cells and then detected by IFA. Sf9 cells infected with recombinant baculovirus

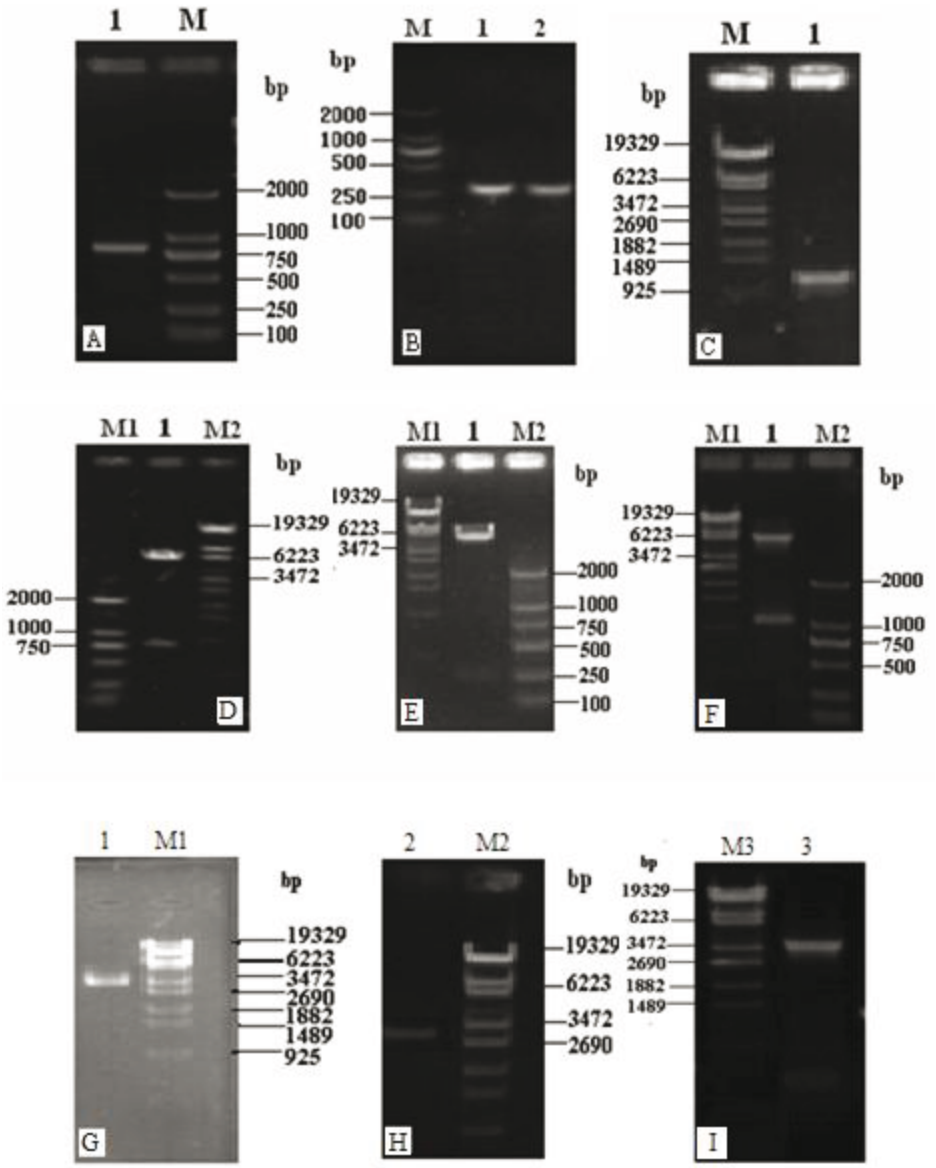

Figure 1. Construction and identification of recombinant bacmid by restriction digestion and electrophoresis. **(A)** Electrophoresis of *M* gene. M, DL-2000 marker; 1, M gene. **(B)** Electrophoresis of *sM* gene. M, DL-2000 marker; 1, 2, *sM* gene. **(C)** Electrophoresis of *N* gene. M, DL-2000 marker; 1, N gene. **(D)** Identification of recombinant plasmid pFastBac Dual-M by restriction digestion. M1, DL-2000 marker; M2, λ-Ecot-14 marker; 1, pFastBac Dual-M digested by *BamH* I and *EcoR* I. **(E)** Identification of recombinant plasmid pFastBac Dual-sM by restriction digestion. M1, λ-Ecot-14 marker; M2, DL-2000 marker; 1, pFastBac Dual-sM digested by *Sma* I and *Kpn* I. **(F)** Identification of recombinant plasmid pFastBac Dual-N by restriction digestion. M1, λ-Ecot-14 marker; M2, DL-2000 marker; 1, pFastBac Dual-N digested by *Sma* I and *Xho* I. **(G)** Identification of recombinant Bacmid-M. M1, λ-Ecot-14 marker; 1, PCR product of recombinant bacmid Bacmid-M. **(H)** Identification of recombinant Bacmid-sM. M2, λ-Ecot-14 marker; 2, PCR product of recombinant bacmid Bacmid-sM. **(I)** Identification of recombinant Bacmid-N. M3, λ-Ecot-14 marker; 3, PCR product of recombinant bacmid Bacmid-N.

showed green fluorescence, but the normal Sf9 cells did not. The results suggested that recombinant proteins were expressed by the baculovirus expression system (Figure 2).

3.3. Assembly of VLPs

To determine if these recombinant proteins could interact with each other and assemble into particulate structures, Sf9 cells were infected with recombinant baculoviruses expressing M, sM, or N alone or coinfected with the combination of M and sM or M and N. Electron microscopic analysis of the negatively stained infected cell sections exhibited no particulate structures when the sM and N proteins were expressed alone; there was only

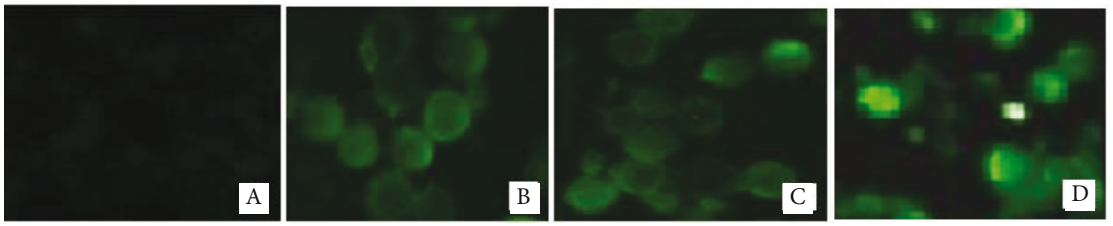

Figure 2. IFA of Sf9 cells infected by recombinant baculovirus rBac-M, rBac-sM, and rBac-N (200×). **(A)** Normal Sf9 insect cells. **(B)** Sf9 insect cells infected by rBac-M. **(C)** Sf9 insect cells infected by rBac-sM. **(D)** Sf9 insect cells infected by rBac-N.

baculovirus virion in infected Sf9 cells. However, cells infected with recombinant viruses expressing M protein alone and coexpressing M and sM proteins could lead to the occurrence of virions morphologically similar to TGEV. The diameter of VLPs was variable from 61 nm to 101 nm, and envelope-like structures appeared in some virions. Coexpression of M and N proteins in insect Sf9 cells infected by recombinant baculovirus could also assemble into VLPs. The results demonstrated that the M protein was sufficient for the assembly of particles (Figure 3).

4. Discussion

The integral membrane protein, the envelope protein (sM), and the nucleocapsid protein (N) are major structural proteins of TGEV. These structural proteins play a vital role in the processes of viral replication, pathogenesis, and assembly. The M protein is mainly embedded in the lipid envelope and its function is similar to that of nonglycosylated membrane protein in rhabdovirus, orthomyxovirus, or paramyxovirus. M protein, containing a high mannose asparagine-linked side chain, is responsible for the connection of the nucleocapsid and envelope, and it plays an important role in the assembly of viral particles by affecting the sites of assembly and budding. The sM protein may regulate the assembly and release of TGEV and is also needed in effective replication of the virus. The nucleocapsid protein is bound up with viral RNA forming the ribonucleoprotein complex in the assembly process of the virus particle, and then it interacts with membrane protein to migrate to the mature region of the viral packaging-ER-Golgi intermediate compartment (18). Currently, morphogenesis of coronavirus containing mouse hepatitis virus (MHV), infectious bronchitis virus (IBV), and severe acute respiratory (SARS) have been reported (19–22). Research results have shown that M protein and sM protein have more effects on the process of viral morphogenesis, and the coronavirus-like particles can be formed by coexpressing M protein and sM protein in mammalian cells (23). The M protein plays a key role in the morphological processes of coronaviruses, and the point mutation in the N-terminus and C-terminus of this protein will significantly affect the assembly of virus particles. In particular, deletion of the last C-terminal amino acid residue could not make M protein participate in the assembly of VLPs, but the stability of virus particles may remain constant (24). In studies on the assembly of IBV and MHV particles, it was found that VLPs were released into the supernatant when IBV sM protein was expressed in BHK-21 alone (15), and the results suggested that even without M protein, the sM protein could promote the formation of VLPs independently. M protein (IBV and MHV) expressed alone cannot generate VLPs, but an envelope-like structure can be observed in infected cells (25). Tseng et al. showed that M protein has the capability of assembling and releasing SARS in 293T cells; spherical particles (100 nm in diameter) were observed in transfected supernatants and M alone formed particles that had densities of 1.13 g/mL, suggesting that M alone is sufficient for particle formation (26). Consistent with our results, the TGEV M is capable of assembling virus particles in the absence of other viral components.

The M protein is an important component of the core structures in TGEV, and the C-terminal amino acid sequence is also essential for the stability of the viral core structures. Baudoux et al. showed that VLPs are released into the culture medium of cells coexpressing M and sM proteins in vitro, and the VLPs formed by M and sM proteins could induce IFN-α to protect from the infection of TGEV (17). In this study, three structural proteins of TGEV were expressed in Sf9 insect cells by the baculovirus expression system. In vitro experiments for VLP assembly of TGEV indicated that expression of M protein alone and coexpression of M plus sM protein and M plus N protein in Sf9 insect cells infected by recombinant baculovirus could result in the occurrence of virions morphologically similar to TGEV. Unlike other coronaviruses (27,28), the M protein of TGEV could be assembled into VLPs just by being expressed in vitro alone. This finding is not in accordance with previous results (17,29,30), and the reason is probably that the M protein of TGEV is different from that of other coronaviruses (such as IBV and MHV) in the assembly of virion particles. Electron microscope analysis, as shown in Figure 3, demonstrated M protein in the form of membrane-enveloped vesicles with densities lower than those of VLPs formed by M plus sM or M plus N. This suggests that TGEV M is not a major determinant of virus

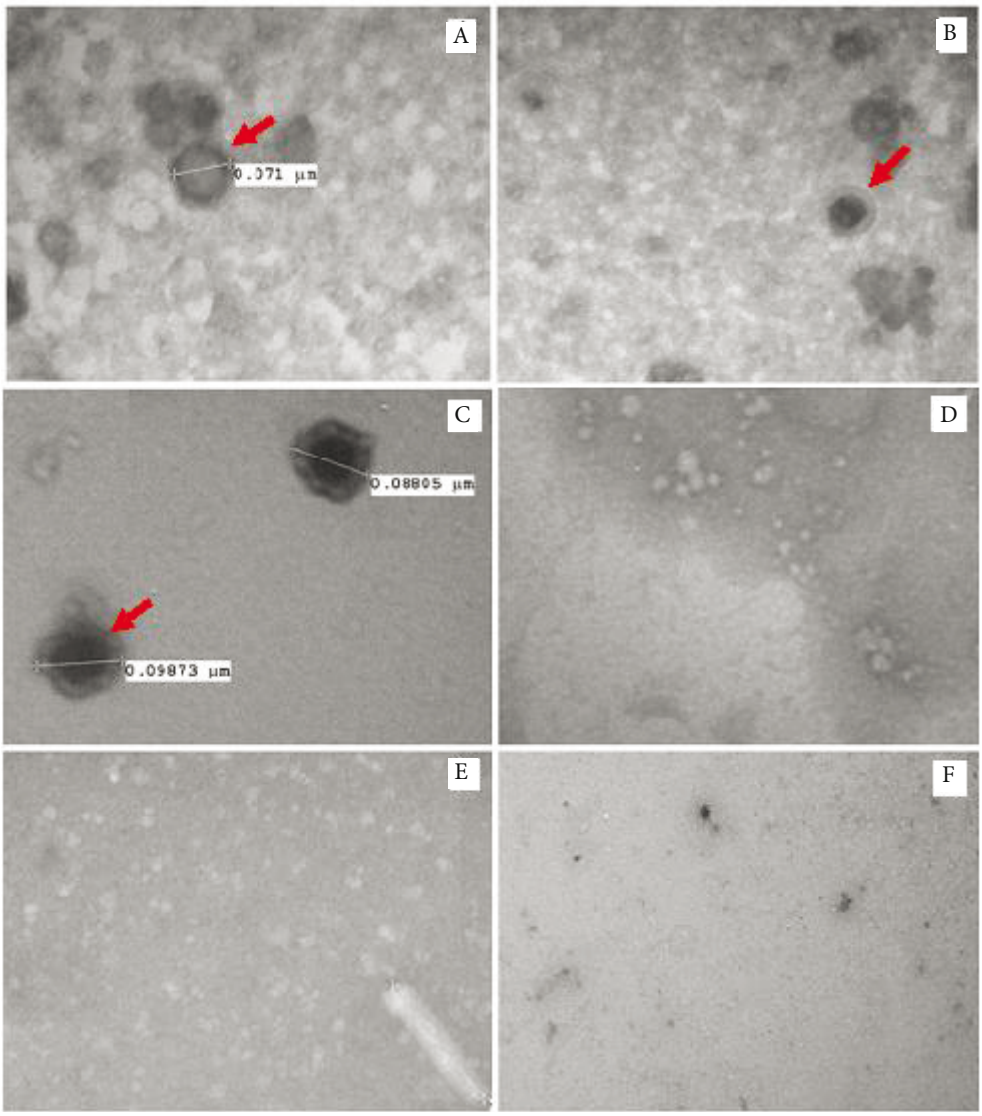

Figure 3. Assembly of virus-like particles by expression of structural proteins of TGEV. **(A)** Negative-staining electron microscopy of virus-like particles formed by expression of recombinant *M* gene. **(B)** Negative-staining electron microscopy of virus-like particles formed by coexpression of recombinant *M*/*sM* genes. **(C)** Negative-staining electron microscopy of virus-like particles formed by coexpression of recombinant *M*/*N* genes. **(D)** Negative-staining electron microscopy of virus-like particles formed by expression of recombinant *sM* gene. **(E)** Negative-staining electron microscopy of virus-like recombinant *N* gene. **(F)** Negative-staining electron microscopy of virus-like normal Sf9 insect cells.

particle density, such as those reported in self-assembly of the SARS M protein (26). Obvious VLPs were not observed in Sf9 cells when sM or N protein was expressed alone by using the baculovirus expression system. Consistent with this result, experiments on the assembly of coronavirus particles have shown that the N protein is a nonessential component in the formation of VLPs (19). Mammals are the natural hosts of TGEV, but there are some subtle differences between baculovirus-insect cell expression systems and mammalian cell expression systems in the process of translation, processing, and transport for protein expression. Therefore, further study is needed to confirm the function and mechanism of TGEV structural protein expressed in mammalian cells for the assembly of VLPs.

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