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Reciprocal complementation of bovine parainfluenza virus type 3 lacking either the membrane or fusion gene

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ABSTRACT

Two defective bovine parainfluenza virus type 3 (BPIV3) strains were generated, one lacking the membrane (M) protein gene and expressing EGFP (ΔM-EGFP) and the other lacking the fusion (F) protein gene and expressing mStrawberry (ΔF-mSB), by supplying deficient proteins *in trans*. When Madin-Darby bovine kidney (MDBK) cells were co-infected with ΔM-EGFP and ΔF-mSB at a multiplicity of infection (MOI) of 0.1, complemented viruses were easily obtained. Complemented viruses grew as efficiently as wild-type BPIV3 and could be passaged in MDBK cell cultures even at an MOI of 0.01, possibly due to multiploid virus particles containing genomes of both ΔM-EGFP and ΔF-mSB. This reciprocal complementation method using two defective viruses would be useful to express large or multiple proteins in cell cultures using paramyxovirus vectors.

1. Introduction

Bovine parainfluenza virus type 3 (BPIV3), a member of the genus *Respirovirus* in the family *Paramyxoviridae* in the order *Mononegavirales*, is an enveloped virus with a non-segmented negative-sense RNA genome (Karron and Collins, 2013). The BPIV3 genome encodes six structural proteins: nucleocapsid (N), phospho (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large (L) proteins. The M protein is a nonintegral, membrane-associated protein localizing under the lipid bilayer of virus particles. The M protein is a key driver of virus particle formation. The F protein is an integral membrane protein on virus particles and essential for virus-cell fusion (Karron and Collins, 2013).

Reverse genetics systems of *Mononegavirales* were first achieved for rabies virus in 1994 (Schnell et al., 1994). Since then, many reverse genetics systems have been established for *Mononegavirales*, and generated recombinant viruses were utilized to study the function of viral proteins or viral genome sequences. Reverse genetics systems of *Mononegavirales* have also been used to generate novel vaccines expressing foreign proteins as antigens (Le Bayon et al., 2013; Sato et al., 2011) or novel medicines such as oncolytic viruses (Pfaller et al., 2015). Recently, recombinant paramyxoviruses are used to establish induced pluripotent stem (iPS) cells (Ban et al., 2011; Nishimura et al., 2011). However, the insertion of multiple or long transcription cassettes of foreign genes into paramyxovirus genomes could reduce viral growth (Bukreyev et al., 2006). To increase the capacity of paramyxovirus vectors to carry multiple or long extra gene units, paramyxoviruses containing segmented genomes were generated (Gao et al., 2008; Takeda et al., 2006).

In this study, we generated two defective BPIV3 strains, one lacking the M protein gene and the other lacking the F protein gene, to increase the capacity of BPIV3 vectors and propagated them by co-infection.

2. Materials and methods

2.1. Cells and viruses

MDBK, HeLa and Vero monolayer cell cultures were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin G and 100 μg/ml of streptomycin. The modified vaccinia virus Ankara (MVA-T7), which expresses the phage T7 RNA polymerase (Wyatt et al., 1995), was grown in chicken embryonic fibroblasts.

2.2. Cloning of the M and F genes of BPIV3 into a pCAGGS plasmid vector

To construct the M-expressing plasmid, the open reading frame of the M gene was synthesized from p(+)BPIV3-EGFP (Ohkura et al.,

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2015) by PCR using the primers 5′-CGCGCTCGAGATGAGCATTACCAACTCTGC-3′ (*Xho*I site is underlined) and 5′-CGCGCGATATCTTACTGTCTGATTTTCCCGA-3′ (*Eco*RV site is underlined) and ligated between *Xho*I and *Eco*RV sites of the pCAGGS plasmid (Niwa et al., 1991), resulting in pCAGGS-M. To construct the F-expressing plasmid, the open reading frame of the F gene was synthesized from p(+)BPIV3-EGFP (Ohkura et al., 2015) by PCR using the primers 5′-GCGCCTCGAGCATGATCATCACAAACACAAT-3′ (*Xho*I site is underlined) and 5′-GCGCGGATATCTCATTGTCTACTTGTTAGTA-3′ (*Eco*RV site is underlined) and ligated between *Xho*I and *Eco*RV sites of pCAGGS plasmid, resulting in pCAGGS-F.

2.3. Construction of BPIV3 cDNA deficient of either the M or F gene

To construct BPIV3 cDNA deficient of the M gene, two fragments were synthesized from p(+)BPIV3-EGFP by PCR. The first fragment covering the *Xho*I site in the P gene to the noncoding region between the gene start sequence for the M gene and M gene open reading frame was amplified using primers 5′-GCAGCTCAGATAGTAGAGCT-3′ and 5′-GCGAAGCTTCGGAGGATGGATTGATACTT-3′ (*Hin*dIII site is underlined) and digested with *Xho*I and *Hin*dIII. The second fragment covering the noncoding region between the gene start sequence for the F gene and F gene open reading frame and the *Nhe*I site in the HN gene was amplified using primers 5′-GCGAAGCTTCCAATACATAG ATCACAGGA-3′ (*Hin*dIII site is underlined) and 5′-GCGGCTAGCCTGATTGCAGTCTCTCTGTG-3′ (*Nhe*I site is underlined) and digested with *Hin*dIII and *Nhe*I. The two PCR fragments were then ligated between *Xho*I and *Nhe*I sites of p(+)BPIV3-EGFP, resulting in p(+)BPIV3ΔM-EGFP. To construct BPIV3 cDNA deficient of the F gene, two fragments were synthesized from p(+)BPIV3-EGFP by PCR. The first fragment covering the *Xho*I site in the P gene to the noncoding region between the gene start sequence for the F gene and F gene open reading frame was amplified using primers 5′-GCAGCTCAGATAGTAGAGCT-3′ and 5′-GCGAAGCTTTAACTGTT GCTCGGAGTTTG-3′ (*Hin*dIII site is underlined) and digested with *Xho*I and *Hin*dIII. The second fragment covering the noncoding region between the gene start sequence for the HN gene and HN gene open reading frame and the *Nhe*I site in the HN gene was amplified using primers 5′-CGGAAGCTTAGAGACGACACCAAATTCAA-3′ (*Hin*dIII site is underlined) and 5′-GCGGCTAGCCTGATTGCAGTCTCTCTGTG-3′ (*Nhe*I site is underlined) and digested with *Hin*dIII and *Nhe*I. The two PCR fragments were then ligated between *Xho*I and *Nhe*I sites of p(+)BPIV3-EGFP, resulting in p(+)BPIV3ΔF-EGFP. The mStrawberry (mSB) gene was synthesized from pmStrawberry (Clontech, Mountain View, CA) by PCR using primers 5′-CCCGTCGACCACCATGGTGAGCAAGGGCGAG-3′ (*Sal*I site is underlined) and 5′-CCCACGCGTTTACTTGT ACAGCTCGTCCATGCC-3′ (*Mlu*I site is underlined) and digested with *Sal*I and *Mlu*I. The EGFP gene in p(+)BPIV3ΔF-EGFP was removed by digesting with *Sal*I and *Mlu*I and replaced with the mSB gene digested with *Sal*I and *Mlu*I, resulting in p(+)BPIV3ΔF-mSB. All plasmids were prepared in Stbl2 cells (Life Technologies, Grand Island, NY) at 30° C.

2.4. Rescue of infectious viruses from p(+)BPIV3ΔM-EGFP or p(+) BPIV3ΔF-mSB plasmids

HeLa cells in a 6-well plate (80% confluent) were infected with vaccinia virus MVA-T7 at an MOI of 1. One hour post-infection, the p(+)BPIV3ΔM-EGFP or p(+)BPIV3ΔF-mSB plasmid (4 μg) was transfected into the MVA-T7-infected HeLa cells together with pCAGGS-M or pCAGGS-F in addition to supporting plasmids (pGEM-N, pGEM-P and pGEM-L) in the presence of 10 μl of Lipofectamine 2000 (Life Technologies) in 250 μl of Opti-MEM (Life Technologies). After 6 h incubation, media were replaced with DMEM supplemented with 10% FBS and antibiotics. Three days post-transfection, the supernatants of p(+)BPIV3ΔM-EGFP- or p(+)BPIV3ΔF-mSB-transfected cells were harvested and transferred onto Vero cells transfected with pCAGGS-M or pCAGGS-F, respectively. After incubation for 3 days, rBPIV3ΔM-EGFP or rBPIV3ΔF-mSB were recovered from p(+)BPIV3ΔM-EGFP- or p(+) BPIV3ΔF-mSB-transfected cells, respectively.

2.5. Reciprocal complementation between M- and F-deficient BPIV3 strains

Monolayer cultures of MDBK cells in 6-well cluster plates were infected with rBPIV3ΔM-EGFP and rBPIV3ΔF-mSB at an MOI of 0.1 50% tissue culture infective dose ($TCID_{50}$)/cell and incubated at 37° C for 3 days.

2.6. Microscopic detection of EGFP and mSB fluorescence

EGFP and mSB fluorescence in infected cells was photographed using a fluorescence microscope (TS100, Nikon, Tokyo, Japan) equipped with a charge-coupled device (CCD) camera (DS-Fi1, Nikon).

2.7. Growth curves

Monolayer cultures of MDBK cells in 24-well cluster plates were infected with the rBPIV3ΔM-EGFP, rBPIV3ΔF-mSB or reciprocally complemented viruses and incubated at 37° C. At various time points, media were harvested and the infectious titer was determined by the $TCID_{50}$ in MDBK cells under a fluorescent microscope.

2.8. Western blotting

Cells were lysed in SDS loading buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and were disrupted by sonication for 10 min. After centrifugation, the lysates were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). The proteins in the gel were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, Mass). M and F proteins were detected using rabbit sera against synthetic peptides corresponding to the M protein (CRSKDRYGSVSDLDDDPS) and the F protein (IQGKNQNDKNSE PYVLTSRQ), respectively.

2.9. Recover of viruses from plaques

Viruses in plaques were recovered with agarose gel overlay using sterilized Pasteur pipets under a fluorescent microscope and were suspended in the medium. After brief centrifugation, viruses were inoculated into MDBK cells.

3. Results

3.1. Generation of an M or F gene-deficient BPIV3 strain

To construct M or F gene-deficient infectious BPIV3 cDNA, the M or F gene was deleted from p(+)BPIV3-EGFP (Ohkura et al., 2015) using mutant primers and RT-PCR, resulting in the generation of p(+)BPIV3ΔM-EGFP or the p(+)BPIV3ΔF-EGFP, respectively. To discriminate M- and F-deficient viruses, the EGFP gene of p(+)BPIV3ΔF-EGFP was replaced with mSB gene, resulting in p(+)BPIV3ΔF-mSB (Fig. 1A). The M-deficient virus (ΔM-EGFP) and the F-deficient virus (ΔF-mSB) were recovered from p(+) BPIV3ΔM-EGFP and p(+)BPIV3ΔF-mSB using reverse genetics for BPIV3 (Ohkura et al., 2015) by supplying the M protein from pCAGGS-M plasmid or the F protein from pCAGGS-F plasmid, respectively. ΔM-EGFP and ΔF-mSB were propagated in Vero cells transfected with pCAGGS-M and pCAGGS-F, respectively. Immunofluorescence assay and western blotting confirmed that ΔM-EGFP and ΔF-mSB expressed EGFP and mSB, respectively (Fig. 1B), and not M protein and F protein, in infected MDBK cells (Fig. 1C). Interestingly, ΔM-EGFP occasionally induced EGFP-expressing satellite cells at late stage (Fig. 1B). ΔM-EGFP may have enhanced cell–cell fusion activity as reported for other M-less paramyxoviruses (Cathomen et al., 1998).