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Replication-competent fluorescent-expressing influenza B virus

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ABSTRACT

Influenza B viruses (IBVs) cause annual outbreaks of respiratory illness in humans and are increasingly recognized as a major cause of influenza-associated morbidity and mortality. Studying influenza viruses requires the use of secondary methodologies to identify virus-infected cells. To this end, replication-competent influenza A viruses (IAVs) expressing easily traceable fluorescent proteins have been recently developed. In contrast, similar approaches for IBV are mostly lacking. In this report, we describe the generation and characterization of replication-competent influenza B/Brisbane/60/2008 viruses expressing fluorescent mCherry or GFP fused to the C-terminal of the viral non-structural 1 (NS1) protein. Fluorescent-expressing IBVs display similar growth kinetics and plaque phenotype to wild-type IBV, while fluorescent protein expression allows for the easy identification of virus-infected cells. Without the need of secondary approaches to monitor viral infection, fluorescent-expressing IBVs represent an ideal approach to study the biology of IBV and an excellent platform for the rapid identification and characterization of antiviral therapeutics or neutralizing antibodies using high-throughput screening approaches. Lastly, fluorescent-expressing IBVs can be combined with the recently described reporter-expressing IAVs for the identification of novel therapeutics to combat these two important human respiratory pathogens.

1. Introduction

Influenza viruses belong to the family *Orthomyxoviridae* and are divided into types A, B, and C (Palese and Shaw, 2007). Despite the use of vaccines, type A and B influenza viruses (IAVs and IBVs, respectively) infect humans regularly and are responsible of yearly seasonal epidemics associated with significant public health and economic consequences (Molinari et al., 2007). It is estimated that, each year, thousands of people worldwide contract influenza and develop acute respiratory infection with significant morbidity and mortality (Luckhaupt et al., 2012; Molinari et al., 2007; Thompson et al., 2003). IAVs are further classified into different subtypes based on the antigenic major surface glycoproteins: hemagglutinin (HA; 18 subtypes) and neuraminidase (NA; 11 subtypes) (Palese and Shaw, 2007; Tong et al., 2012; Tong et al., 2013). Unlike IAVs, IBVs are not divided into antigenically distinct subtypes, although since the 1980s two lineages diverged from the ancestral influenza virus B/Lee/1940 strain and have been co-circulating in the human population (Chen and Holmes, 2008; McCullers et al., 2004). Currently, only H3N2 and H1N1 IAV subtypes and IBVs circulate in humans (Shaw and Palese, 2013; Tong et al., 2012; Tong et al., 2013). IAVs and IBVs follow a rather diffuse cyclical epidemic pattern based on prevalence, typical once every 3 years (Hite et al., 2007; Li et al., 2008; Lin et al., 2004; Olson et al., 2007). IBV epidemics tend to be less severe than H3N2 IAVs but more severe than H1N1 AIVs in adults and the elderly (Ohmit and Monto, 1995; Olson et al., 2007; Thompson et al., 2003; Van Voris et al., 1982) However, IBV infections are associated with excess morbidity and mortality in the pediatric population (Belshe, 2010; Hite et al., 2007; Li et al., 2008; Olson et al., 2007). Contrary to IAVs, which has a broad host reservoir in many avian and mammalian species, IBVs are mainly restricted to humans (Wright et al., 2007), although occasional infections of seals have been documented (Osterhaus et al., 2000).

Vaccines and antivirals are available to combat influenza viruses (Baker et al., 2015b; Burnham et al., 2013; Jackson et al., 2011a; Krammer et al., 2015; Nguyen et al., 2010; Seibert et al., 2010). Historically, influenza vaccines contain viral antigens corresponding to the prevalent H3N2 and H1N1 IAVs as well as and one lineage of

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influenza type B (Victoria or Yamagata) (Belshe et al., 2007; Yang, 2013). More recently and due to increasing co-circulation of both IBV lineages with significant antigenic divergence, the Advisory Committee on Immunization Practices (ACIP) recommended that influenza vaccines should be available in quadrivalent formulations (Grohskopf et al., 2014; Sun, 2012). With respect to antivirals, there are four classes of FDA-approved drugs for use against influenza infections. Rimantadine and amantadine that target the viral matrix 2 (M2) ion channel and inhibit viral entry (Hay et al., 1985) but are not effective against the M2 protein of IBVs (Beigel and Bray, 2008). Zanamivir and oseltamivir target the sialidase activity of the influenza virus NA and inhibit virus release and are effective against both IAVs and IBVs (Jackson et al., 2011b). Low clinical effectiveness of NA inhibitors against IBVs in children and the emergence of drug resistant variants during treatment has been reported (Burnham et al., 2013; Farrukee et al., 2013).

Plasmid-based reverse genetics to generate recombinant influenza viruses (Fodor et al., 1999; Martinez-Sobrido and Garcia-Sastre, 2010; Neumann et al., 1999) has significantly contributed to a better understanding of the biology of these important human respiratory pathogens (Engelhardt, 2013; Jackson et al., 2011a), for the identification and characterization of antivirals (Baker et al., 2014; Ozawa and Kawaoka, 2011; Roberts et al., 2015), and for the development of alternative influenza vaccines (Baker et al., 2015a; Martinez-Sobrido and Garcia-Sastre, 2010; Nogales et al., 2014a; Subbarao and Katz, 2004). Reverse genetics have allowed for the generation of replication-competent IAVs expressing reporter genes as novel powerful tools to track viral infections without the requirement of secondary methodologies to detect viral-infected cells (Eckert et al., 2014; Fiege and Langlois, 2015; Fukuyama et al., 2015; Kittel et al., 2004; Manicassamy et al., 2010; Nogales et al., 2014a; Pan et al., 2013; Perez et al., 2013; Reuther et al., 2015; Tran et al., 2013). In contrast, similar approaches have not been implemented to the same extend for IBV. Here we describe and characterize recombinant IBVs based on the B/Brisbane/60/2008 (Victoria lineage) strain, where fluorescent proteins (mCherry or GFP) were fused to the C-terminal end of the viral NS1 (Nogales et al., 2014a). Fluorescent-expressing IBVs have similar growth kinetics and plaque phenotype than the respective wild-type (WT) counterpart. Importantly, IBV infections were easily tracked in real-time using fluorescence microscopy and conveniently quantified using a fluorescent plate reader. By eliminating the use of secondary approaches to monitor IBV infections, these replication-competent, fluorescent-expressing IBVs represent a promising option to study the biology of IBV and an excellent platform to easily identify and evaluate antivirals or neutralizing antibodies (NAbs) using high-throughput screening (HTS) approaches, both currently needed to combat this important human respiratory pathogen.

2. Materials and methods

2.1. Cell lines

Human embryonic kidney 293T (ATCC CRL-11268) and canine Madin-Darby canine kidney (MDCK; ATCC CCL-34) cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% PSG (penicillin, 100 units/ml; streptomycin 100 μg/ml; L-glutamine, 2 mM) (Nogales et al., 2014b).

2.2. Construction of the NS plasmids

To engineer an IBV NS segment where the C-terminal of NS1 is fused to a fluorescent mCherry or codon-optimized maxGFP (GFP; Amaxa), a recombinant NS segment was synthesized *de novo* (Biomatik) with appropriate restriction sites for subcloning into the ambisense plasmid pDP-2002 (Pena et al., 2013). This plasmid, named pDP-NS-2xBsmBI, contained the NS1 open reading frame (ORF), without the stop codon or splice acceptor site, and two BsmBI sites in opposite orientation followed by the porcine teschovirus-1 (PTV-1) 2A autoproteolytic cleavage site (ATNFSLLKQAGDVEENPGP) and the entire sequence of the nuclear export protein, NEP (Nogales et al., 2014a). The mCherry and GFP ORFs were amplified by PCR using oligonucleotides designed to introduce complementary BsmBI sites, and then cloned into the pDP-NS-2xBsmBI to generate the pDP-NS-mCherry and pDP-NS-GFP plasmids for IBV rescues. Oligonucleotides for the cloning of mCherry and GFP into the pDP-NS-2xBsmBI plasmid are available upon request. Plasmid constructs were confirmed by sequencing (ACGT, Inc.).

2.3. Rescue of recombinant fluorescent-expressing viruses and viral infections

Ambisense pDP-2002 plasmids were used for the rescue of WT and reporter-expressing B/Brisbane/60/2008 as previously described (Baker et al., 2014; Pena et al., 2013). Briefly, co-cultures (1:1) of 293T/MDCK cells (6-well plate format, 10^6 cells/well) were co-transfected in suspension with the eight ambisense pDP-2002-PB2, -PB1, -PA, -HA, -NP, -NA, -M and WT NS, NS-mCherry, or NS-GFP plasmids. Clonal WT, mCherry- or GFP-expressing IBVs were selected by plaque assay and the virus stocks were propagated in MDCK cells at 33 °C in a 5% CO_2 atmosphere for 3-4 days. Influenza A/California/04/2009H1N1 (pH1N1) WT and mCherry viruses have been previously described (Baker et al., 2013; Nogales et al., 2014a). For infections, virus stocks were diluted in phosphate buffered saline (PBS) supplemented with 0.3% bovine albumin (BA) and 1% PS (PBS/BA/PS). After viral infections, cells were maintained in DMEM supplemented with 0.3% BA, 1% PSG, and 1 μg/ml tosyl-sulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) (Martinez-Sobrido and Garcia-Sastre, 2010). Virus titers were determined by standard plaque assay (plaque forming units (PFU)/ml) in MDCK cells (Nogales et al., 2014b).

2.4. Virus growth kinetics and titrations

Multicycle virus growth kinetics were performed in MDCK cells (12-well plate format, 5×10^5 cells/well) infected with the indicated viruses (triplicates) at multiplicity of infection (MOI) of 0.001. Virus titers in the tissue culture supernatants were determined by immunofocus assay (fluorescent focus-forming units, FFU/ml) (Nogales et al., 2014b) using mouse monoclonal antibodies (MAbs) against IAV (HT103) (O'Neill et al., 1998) or IBV (B017; Abcam) NP, and a fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (Dako). Mean value and standard deviation (SD) were calculated using Microsoft Excel software.

2.5. Protein gel electrophoresis and Western blots

Cell extracts from either mock or virus-infected (MOI 1.0) MDCK cells (6-well plate format, 10^6 cells/well) were lysed at 18 h post-infection (hpi) in passive lysis buffer (Promega) and separated by denaturing electrophoresis as previously described (Nogales et al., 2014b). Membranes were blocked for 1 h with 5% dried skim milk in PBS containing 0.1% Tween 20 (T-PBS) and incubated overnight at 4 °C with the indicated primary monoclonal or polyclonal (PAb) antibodies against IBV NP (mouse MAb B017; Abcam), mCherry (rabbit PAb; Raybiotech), IBV NS1 (rabbit PAb; kindly provided by Dr. T. Wolff) (Dauber et al., 2006) or actin (mouse MAb; Sigma). Bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibodies (GE