Virology 497 (2016) 33–40

Contents lists available at ScienceDirect

Virology

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

Enhancing dengue virus maturation using a stable furin over-expressing cell line

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ARTICLE INFO

Article history:
Received 31 March 2016
Returned to author for revisions
25 June 2016
Accepted 27 June 2016
Available online 13 July 2016

Keywords:
Flavivirus
Dengue virus
West Nile virus
Virion maturation
Antibody neutralization
Furin

ABSTRACT

Flaviviruses are positive-stranded RNA viruses that incorporate envelope (E) and premembrane (prM) proteins into the virion. Furin-mediated cleavage of prM defines a required maturation step in the flavivirus lifecycle. Inefficient prM cleavage results in structurally heterogeneous virions with unique antigenic and functional characteristics. Recent studies with dengue virus suggest that viruses produced in tissue culture cells are less mature than those produced in primary cells. In this study, we describe a Vero cell line that ectopically expresses high levels of human furin (Vero-furin) for use in the production of more homogenous mature flavivirus populations. Laboratory-adapted and clinical dengue virus isolates grow efficiently in Vero-furin cells. Biochemical and structural techniques demonstrate efficient prM cleavage in Vero-furin derived virus preparations. These virions also were less sensitive to neutralization by antibodies that bind efficiently to immature virions. This furin-expressing cell line will be of considerable utility for flavivirus neutralization and structural studies.

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1. Introduction

Flaviviruses are enveloped positive-stranded RNA viruses transmitted principally by arthropod vectors. Many viruses in this genus, such as West Nile (WNV), dengue (DENV), and Zika viruses, have a considerable impact on global health due to their potential for rapid emergence and morbidity (Fauci and Morens, 2016; Guzman and Harris, 2015; Mackenzie et al., 2004). Flavivirus virions assemble on membranes of the endoplasmic reticulum as immature virus particles that incorporate two viral structural proteins on their surface (Mackenzie and Westaway, 2001; Welsch et al., 2009; Zhang et al., 2003). The envelope (E) protein is an elongated three-domain class II fusion protein tethered to the viral membrane by a helical stem and two transmembrane helices (Zhang et al., 2013a). It is responsible for binding to cellular attachment factors (Perera-Lecoin et al., 2014) and promoting fusion of viral and cellular membranes following endocytosis and exposure to a low pH environment (Sanchez-San Martin et al., 2009). The premembrane (prM) protein associates with E shortly after synthesis (Lorenz et al., 2002) and is incorporated into virus particles as a heterotrimer. Each immature virion contains sixty heterotrimeric spikes arranged in an icosahedral fashion (Zhang et al., 2003, 2007). In this configuration, E proteins cannot undergo changes in conformation required for viral fusion, rendering immature particles non-infectious (Guirakhoo et al., 1991; Zybert et al., 2008). Conversion of immature virions to an infectious form occurs while virus particles traffic through the secretory pathway. In the low pH environment of the trans-Golgi network, immature virions undergo a structural transition that exposes on prM a site recognized by host furin-like proteases (Li et al., 2008; Stadler et al., 1997; Yu et al., 2008). Cleavage of prM is the defining event in flavivirus maturation and is a required step in the virus infection cycle (Elshuber et al., 2003; Stadler et al., 1997). The products of this cleavage event are a membrane anchored nine kDa peptide (the membrane (M) protein) and a soluble "pr" fragment (~22 kDa) that disassociates from the virion upon release from the cell (Yu et al., 2009). The E proteins on mature virions are arranged as antiparallel dimers arrayed in a herringbone fashion (Kuhn et al., 2002; Zhang et al., 2013a).

Flaviviruses are secreted from cells as a heterogeneous mixture of virions due in part to inefficiency of the prM cleavage reaction

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http://dx.doi.org/10.1016/j.virol.2016.06.022
0042-6822/Published by Elsevier Inc.

(reviewed in (Pierson and Diamond, 2012)). Partially mature virions are defined herein as having structural features of both mature and immature virus particles. Structural studies suggest that the E proteins of partially mature virions are arranged as mosaics of immature virus-like prM-E heterotrimers and antiparallel E dimers, in varying proportions (Plevka et al., 2011). The fraction of virions that retain uncleaved prM *in vitro* and *in vivo*, and the distribution of uncleaved prM among individual virions, are unclear and may vary among cell types (Randolph et al., 1990). Studies of DENV revealed that ~90% of the E protein in C6/36 insect cell-derived stocks of virus can be immunoprecipitated with a prM-reactive antibody (Junjhon et al., 2010); these studies suggest prM+ virions may be common. Partially mature virions may be infectious, although the extent of cleavage required for virus infectivity remains unknown (Pierson and Diamond, 2012). Incomplete prM cleavage has a significant impact on antibody recognition of infectious viruses (Dowd et al., 2014; Mukherjee et al., 2014a; Nelson et al., 2008). Studies of several flaviviruses have identified neutralizing antibodies that bind E protein epitopes not predicted to be accessible for recognition on the mature virion (Austin et al., 2012; Cherrier et al., 2009; Cockburn et al., 2012; Lok et al., 2008; Oliphant et al., 2006; Stiasny et al., 2006). In many cases, decreasing the efficiency of prM cleavage markedly increases neutralization potency through changes in epitope accessibility. Uncleaved prM also may be bound directly by antibodies with a limited capacity to neutralize infection. In the case of DENV, occupancy by prM-reactive antibodies below the neutralization threshold allows antibody-dependent enhancement of infection (ADE) of Fc-receptor expressing cells (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010), which has the potential to contribute to disease severity (Guzman et al., 2013).

The structural heterogeneity of flaviviruses complicates detailed characterization of structure-function relationships, insights into antibody recognition and neutralization potency, and the production of uniform stocks of live-attenuated vaccine candidates. In this study, we describe the creation and utility of a Vero cell line modified to ectopically express high levels of human furin. Flaviviruses produced in these cells are considerably less heterogeneous than stocks prepared in the parental Vero cell line. These virus preparations are characterized by increased specific infectivity and decreased sensitivity to neutralization by maturation state-dependent neutralizing antibodies, thus confirming the more efficient maturation of infectious virus particles. That both laboratory-adapted and primary DENV strains grow efficiently on this cellular substrate provides a powerful new tool for the study of flavivirus structure and humoral immunity. Because primary myeloid cells appear to produce more mature virions than cell culture adapted lines (Dejnirattisai et al., 2015), virions derived from Vero-furin cells may have greater relevance for studies of the antigenic relatedness of viruses (Katzelnick et al., 2015), and facilitate efforts to identify *in vitro* assays that predict protection following vaccination.

2. Materials and methods

2.1. Cells

HEK-293T and Vero cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing Glutamax supplemented with 100 U/ml penicillin-streptomycin (PS)(Invitrogen) and 7% fetal bovine serum (FBS)(HyClone). Vero and HEK-293T cell lines that over-express human furin (Vero-furin and 293T-furin, respectively) were cultured in the complete DMEM formulation detailed above supplemented with 10 μg/ml or 5 μg/ml of blasticidin, respectively (Invitrogen). Raji cells that stably express DC-SIGNR (Raji-DCSIGNR) were maintained in RPMI-1640 medium supplemented with 7% FBS and 100 U/ml PS. All mammalian cell culture was performed at 37 °C in the presence of 7% CO_2. The insect cell line C6/36 was cultured in complete DMEM supplemented with 1X non-essential amino acids (Invitrogen), and maintained at 28 °C in the presence of 7% CO_2.

2.2. Stable cell line production

A bicistronic vector (pFIRB) encoding human furin and the blasticidin resistance gene ($blast^R$) was constructed using standard molecular cloning techniques. Briefly, pFIRB was constructed by introducing a 966-bp PCR amplicon encoding the foot-and-mouth disease virus internal ribosome entry site (IRES) and $blast^R$ from a previously described WNV replicon plasmid (Pierson et al., 2006) into a human furin-expressing pcDNA3.1 vector (Davis et al., 2006). pFIRB was transfected into Vero cells and cultured in the presence of 10 μg/ml of blasticidin. Furin-expressing clones were isolated by limiting dilutions and screened for expression by Western blot, as detailed below, and flow cytometry using a furin-specific polyclonal antibody and AlexaFluor 647 labeled secondary antibody (Invitrogen).

2.3. Viruses

An infectious clone of WNV lineage I (strain NY1999) that expresses GFP following infection has been described previously. Virus production using this clone was performed as described previously (Lin et al., 2012). Infectious clones of DENV1 (Western Pacific strain), DENV2 (New Guinea C strain), DENV3 (Sleman strain) and DENV4 (814669 strain) were obtained from Dr. Stephen Whitehead (NIH, Bethesda, MD). DENV was produced by *in vitro* transcription and capped using AmpliCap SP6 Message Maker technology (Epicentre Technologies) according to manufacturer's instructions. Purified viral RNA was transfected into C6/36 cells using the DOTAP liposomal transfection reagent (Roche) in HEPES-buffered saline (pH 7.6). DENV was harvested 5 d post-transfection, passed through a 0.22 μm filter, aliquotted, and frozen at −80 °C until use. Three independent working stocks of each virus were produced by passage in C6/36 cells for use in experiments described within. Clinical Nicaraguan DENV2 and DENV4 viruses were a kind gift from Dr. Eva Harris, University of California, Berkeley. Propagation of the DENV2 16681 for structural studies is detailed below. To reduce the efficiency of prM cleavage in virus preparations, cells were cultured in the presence of 50 μM furin inhibitor Dec-RVKR-CMK (Enzo Life Sciences), as described previously (Mukherjee et al., 2014a, 2011).

2.4. Reporter virus particle (RVP) production

WNV RVPs were produced by complementation of a WNV subgenomic lineage II replicon with WNV structural genes as described (Mukherjee et al., 2014b; Pierson et al., 2006). The replicon used in these studies expresses a GFP reporter, allowing infection to be tracked by flow cytometry.

2.5. Large scale production and purification of DENV2 16681

Vero and Vero-furin cells were grown in 2- and 10-chambered cell stacks (Corning, New York) in DMEM as detailed above. Cells were infected at 80% confluence (~3×10^9 cells) with DENV2 16681 at a multiplicity of infection (MOI) of 0.1. Cells were gently agitated at room temperature for 2 h after which they were overlaid with DMEM containing 2% FBS and incubated further. Culture supernatant was harvested and replaced with fresh media on days 3, 4, 5, and 6 post-infection. Virus containing supernatants

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