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The Zika virus envelope protein glycan loop regulates virion antigenicity

Leslie Goo[a,1], Christina R. DeMaso[a], Rebecca S. Pelc[a,2], Julie E. Ledgerwood[b], Barney S. Graham[b], Richard J. Kuhn[c], Theodore C. Pierson[a,*]

[a] Viral Pathogenesis Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, United States of America
[b] Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA
[c] Markey Center for Structural Biology and Purdue Institute of Inflammation, Immunology and Infectious Disease, Purdue University, West Lafayette, IN, USA

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ABSTRACT

Because antibodies are an important component of flavivirus immunity, understanding the antigenic structure of flaviviruses is critical. Compared to dengue virus (DENV), the loop containing the single N-linked glycosylation site on Zika virus (ZIKV) envelope (E) proteins extends further towards the DII fusion loop (DII-FL) on neighboring E proteins within E dimers on mature viruses. Although ZIKV is poorly neutralized by DII-FL antibodies, we demonstrated significantly increased neutralization sensitivity of ZIKV particles incorporating the DENV glycan loop. Increased neutralization sensitivity was independent of E protein glycosylation: ZIKV lacking E protein glycans remained poorly neutralized, whereas ZIKV loop chimeras with or without an E protein glycan were potently neutralized. ZIKV particles lacking the E protein glycan were capable of infecting Raji cells expressing the lectin DC-SIGNR, suggesting the prM glycan of partially mature particles can facilitate entry. Our study provides insight into the determinants of ZIKV E protein function and antigenicity.

1. Introduction

Flaviviruses are a group of positive stranded RNA viruses typically transmitted to humans via the bite of an infected tick or mosquito. Many members of the *Flavivirus* genus, including dengue virus (DENV), West Nile virus (WNV), and Zika virus (ZIKV) are pathogens of significant public health concern. For example, the four DENV serotypes cause an estimated 390 million infections annually (Bhatt et al., 2013). Flaviviruses are also emerging threats to global health, as exemplified by the introduction of WNV into the United States in 1999 (Nash et al., 2001), and the emergence and rapid spread of ZIKV in the Americas following its introduction into Brazil (Campos et al., 2015; Weaver et al., 2016). While there are no licensed vaccines or therapeutic agents to prevent or treat infections by emerging flaviviruses such as WNV and ZIKV, vaccines are available for several related flaviviruses, including Yellow Fever virus (YFV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and recently, DENV, although with limited efficacy (Guy et al., 2016). Neutralizing antibodies (NAbs), which bind to the surface of the virus to directly inhibit infectivity, are a correlate of protection for many of these vaccines (Belmusto-Worn et al., 2005; Heinz et al., 2007; Markoff, 2000).

Due to the importance of antibodies in flavivirus immunity and the sudden emergence of ZIKV from relative obscurity to a considerable public health threat, current efforts are focused on understanding the antigenic structure of ZIKV to inform vaccine development (Heinz and Stiasny, 2017; Pierson and Graham, 2016), as well as the basis of the apparent distinctive biology of recent ZIKV infections and their resulting clinical syndromes (Lazear and Diamond, 2016). Although historically ZIKV infection was characterized by a mild, self-limiting febrile illness typical of other flavivirus infections, contemporary ZIKV outbreaks have also been associated with serious clinical complications, including congenital microcephaly in infants born to women infected during pregnancy (Brasil et al., 2016; Cauchemez et al., 2016; Honein et al., 2017), and Guillain-Barré syndrome in adults (Cao-Lormeau et al., 2016). Moreover, ZIKV has been detected in urine (Gourinat et al., 2015), saliva (Musso et al., 2015), and semen (Atkinson et al., 2016), and unlike other flaviviruses, sexual transmission of ZIKV has been documented (Foy et al., 2011; Venturi et al., 2016).

The main target of NAbs against flaviviruses is the envelope (E) protein, which mediates entry into cells, and consists of three structural ectodomains (DI, DII, DIII), a helical stem, and a transmembrane region. NAbs against flaviviruses target epitopes in all three structural domains of the E protein, as well as quaternary epitopes that span multiple E proteins (VanBlargan et al., 2016). Conservation among

* Corresponding author.
E-mail address: piersontc@mail.nih.gov (T.C. Pierson).
[1] Present address: Chan Zuckerberg Biohub, San Francisco, CA, USA.
[2] Present address: North Carolina State Laboratory of Public Health, North Carolina Department of Health and Human Services, Raleigh, NC, USA.

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flavivirus E proteins, particularly within the DII fusion loop (DII-FL) results in considerable cross-reactivity among members of the genus. While cross-reactive antibodies targeting DII-FL are not typically highly neutralizing (VanBlargan et al., 2016), two classes of quaternary epitope-specific monoclonal antibodies (mAbs) targeting the E protein dimer interface potently cross-neutralize not only all four DENV serotypes, but also ZIKV (Barba-Spaeth et al., 2016; Dejnirattisai et al., 2015; Swanstrom et al., 2016).

Structural and functional studies reveal that flavivirus virions are dynamic and heterogeneous particles that undergo major conformational and organizational changes during multiple steps of the virus lifecycle. Flaviviruses assemble on the endoplasmic reticulum as non-infectious immature particles that incorporate heterotrimeric spikes composed of E and the pre-membrane (prM) structural protein (Prasad et al., 2017; Zhang et al., 2003, 2007). Virus maturation is a required step in the viral replication cycle defined by pH-dependent cleavage of prM by cellular furin-like proteases during viral egress (Li et al., 2008; Yu et al., 2008), and results in a relatively smooth mature virion on which E proteins are arrayed as 90 antiparallel dimers that lie flat against the viral membrane (Kostyuchenko et al., 2016; Kuhn et al., 2002; Mukhopadhyay et al., 2003; Sirohi et al., 2016; Zhang et al., 2013). However, prM cleavage is often inefficient, resulting in a heterogeneous population of infectious virions that retain varying amounts of uncleaved prM (Pierson and Diamond, 2012). Partially mature virions may differentially interact with antibodies due to differences in epitope accessibility among E proteins arranged as dimers vs. trimers (Cherrier et al., 2009; Nelson et al., 2008). The antigenic structure of flaviviruses is also governed by the conformational dynamics of the virion that results in an ensemble of conformations at equilibrium. This “viral breathing” has the potential to transiently alter the accessibility of otherwise poorly exposed epitopes. Consequently, the neutralization potency of many antibodies can be improved by increasing either the temperature or time of virus-antibody incubation (Dowd et al., 2011). The structural ensembles sampled by flaviviruses may also be impacted by amino acid variation in the structural proteins (Dowd et al., 2015; Goo et al., 2017).

The cryo-electron microscopy (cryo-EM) structure of the mature ZIKV particle revealed many similarities with that of DENV (Kostyuchenko et al., 2016; Sirohi et al., 2016). One significant difference between the structures of these mature virions is the conformation and length of a loop bearing the N-linked glycosylation site at E DI residue ZIKV N154 or DENV N153. Because the presence of E protein glycans has been shown to mediate flavivirus attachment to target cells expressing lectin attachment factors (Davis et al., 2006a, b) and to contribute to WNV pathogenesis (Beasley et al., 2005), this region could play a role in ZIKV infection and pathogenesis. The flavivirus E DI N-linked glycan has also been proposed to contribute to E dimer stabilization via interactions with the adjacent DII-FL on the neighboring E protein within the antiparallel E dimer (Rey et al., 1995). Thus, it is possible that this glycan and the surrounding loop residues may directly modulate DII-FL accessibility.

Here, we investigated the antigenicity of ZIKV particles on which the E protein glycan loop residues were replaced with those of DENV strains representing serotypes 1 and 2. These chimeric particles displayed increased sensitivity to neutralization by antibodies targeting epitopes that include the poorly accessible DII-FL. Antibodies that bind determinants predicted to be more accessible on the mature virion were impacted by the glycan loop substitution to a lesser degree, consistent with prior studies relating changes in antibody potency arising from “viral breathing” to epitope exposure (Dowd et al., 2011). These findings provide a first step into understanding the molecular determinants of ZIKV antigenicity.

2. Materials and methods

2.1. Cell culture

HEK-293T/17 (ATCC) and Vero (ATCC) cells were maintained in Dulbecco's Modified Eagle medium (DMEM) containing 25 mM HEPES (Invitrogen) supplemented with 7% fetal bovine serum (FBS; Invitrogen) and 100 U/ml penicillin-streptomycin (P/S; Invitrogen). Raji B lymphoblast cells (ATCC) engineered to stably express DC-SIGNR (Raji-DCSIGNR) (Davis et al., 2006b; Dowd et al., 2011; Nelson et al., 2008; Pierson et al., 2007) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing Glutamax (Invitrogen) supplemented with 7% FBS and 100 U/ml P/S. All cells were maintained at 37 °C in the presence of 7% CO_2.

2.2. Plasmids

The following sequences were synthesized and cloned into pcDNA3.1– (Genscript): 1) Structural gene (C, prM, E) variant of ZIKV strain H/PF/2013 (Genbank # KJ776791.2) encoding a T156A amino acid mutation in the E protein to abolish the glycosylation site at N154; 2) Structural gene (C, prM, E) variant of ZIKV strain H/PF/2013 in which the nucleotides encoding 20 amino acid residues that form a loop including E residue N154 were replaced with the corresponding sequence surrounding N153 in the DENV serotype 1 strain 16007 (Genbank # AF18018.1) or the DENV serotype 2 strain 16681 (Genbank M84727.1); 3) Structural gene (C, prM, E) variant of DENV1 16007 or DENV2 16681 in which the nucleotides encoding 14 amino acid residues surrounding E residue N153 was replaced with the corresponding sequence surrounding N154 in ZIKV H/PF/2013. Variants of sequences 2) and 3) encoding a mutation at E amino acid residue T155A or T156A that abolishes the glycosylation site at E residue 153 or 154 of DENV and ZIKV, respectively, were similarly synthesized and cloned. A schematic illustration of the amino acids present in each chimera and variant is included in Fig. 1.

2.3. Production of RVPs

RVPs were produced by complementing a GFP-expressing WNV subgenomic replicon with plasmids encoding flavivirus structural gene variants, as described previously (Pierson et al., 2006). Briefly, HEK-293T/17 cells pre-plated in a low-glucose (1 g/liter) formulation of DMEM containing 25 mM HEPES (Invitrogen), 7% FBS, and 100 U/ml P/S were transfected with plasmids encoding the replicon and structural genes at a 1:3 ratio by mass using Lipofectamine 3000 (Invitrogen) and incubated at 37 °C. Four hours post-transfection, cells were transferred to 30 °C. Supernatants harvested at days 3 and 4 post-transfection were pooled, passed through a 0.22 μm filter (Millipore), and stored at –80 °C. To prepare mature RVPs with increased efficiency of prM cleavage, RVPs were produced by co-transfecting plasmids encoding the replicon, structural genes, and human furin at a 1:3:1 ratio.

2.4. Determination of RVP titer

Clarified virus supernatant was serially diluted two-fold in a volume of 100 μl and used to infect 5×10^4 Raji-DCSIGNR cells in a similar volume at 37 °C for two days. Cells were then fixed in 1.8% paraformaldehyde, and GFP-positive cells enumerated by flow cytometry (BD FACSCalibur). Virus titer was calculated using the linear portion of the resulting virus-dose infectivity curve using the following formula: Infectious units (IU)/virus volume = (% GFP-positive cells) x (number of cells) x (dilution factor).

2.5. Monoclonal antibodies and serum samples

Mouse mAbs ZV-13, ZV-48, and ZV-67 were isolated following