Virology 441 (2013) 114–125

Contents lists available at SciVerse ScienceDirect

Virology

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

Mutation of the dengue virus type 2 envelope protein heparan sulfate binding sites or the domain III lateral ridge blocks replication in Vero cells prior to membrane fusion

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ARTICLE INFO

Article history:
Received 11 December 2012
Returned to author for revisions
11 January 2013
Accepted 16 March 2013
Available online 6 April 2013

Keywords:
Dengue virus
Flavivirus
Envelope protein
Attachment
Heparan sulfate
Mutagenesis

ABSTRACT

Using an infectious cDNA clone we engineered seven mutations in the putative heparan sulfate- and receptor-binding motifs of the envelope protein of dengue virus serotype 2, strain 16681. Four mutant viruses, KK122/123EE, E202K, G304K, and KKK305/307/310EEE, were recovered following transfection of C6/36 cells. A fifth mutant, KK291/295EE, was recovered from C6/36 cells with a compensatory E295V mutation. All mutants grew in and mediated fusion of virus-infected C6/36 cells, but three of the mutants, KK122/123EE, E202K, G304K, did not grow in Vero cells without further modification. Two Vero cell lethal mutants, KK291/295EV and KKK307/307/310EEE, failed to replicate in DC-SIGN-transformed Raji cells and did not react with monoclonal antibodies known to block DENV attachment to Vero cells. Additionally, both mutants were unable to initiate negative-strand vRNA synthesis in Vero cells by 72 h post-infection, suggesting that the replication block occurred prior to virus-mediated membrane fusion.
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Introduction

Dengue virus (DENV), a member of *Flaviviridae* family, contains a positive-strand (+) RNA genome and three structural proteins: envelope (E), membrane (M), and capsid (C). The E protein assembles in the virion envelope as homodimer rafts, and mediates viral entry into cells via attachment, endocytosis, and fusion with endosomal membranes—delivering the capsid into the cell cytoplasm. The E protein folds into three major domains. Domain I (DI) is the central domain containing the protein "hinge" responsible for the low pH-catalyzed conformational change from homodimers to fusion-competent homotrimers. Domain II (DII) is the dimerization domain containing the fusion peptide at its tip. Domain III (DIII) is an immunoglobulin-like structure suggested to mediate binding of virus to cellular receptors (Kuhn et al., 2002; Modis et al., 2003, 2005; Mukhopadhyay et al., 2005; Nayak et al., 2009).

DENV replication begins with the attachment of virus to cellular receptors. It is now well established that cell surface glycosaminoglycans (GAGs), such as heparan sulfate (HS), can facilitate flaviviral attachment to cultured mammalian cells (Chen et al., 1997; Germi et al., 2002; Hilgard and Stockert, 2000; Hung et al., 1999). After solving the crystal structures of DENV2 and DENV3 E proteins, Modis et al. (2003, 2005) suggested that three clusters of positively charged amino acids (AAs) on E protein homodimer surface might be involved in HS binding. Cluster 1, located in DI, consists of R188, H282, K284, R286, K288, K291, and K295 (based on DENV2 E protein residue numbers) and all of these are conserved as basic residues among all 4 DENV serotypes and the Japanese encephalitis virus (JEV) complex, including West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), and St. Louis encephalitis virus (SLEV). Cluster 2, located in the middle of DII near the dimer interface, includes K58, K64, K89, K122, K123, and K128 for DENV2. Unlike cluster 1, cluster 2 is less conserved among the DENV or JEV serocomplex viruses, and DENV2 contains more basic residues in this cluster than other DENV serotypes. The moderately variable cluster 3 resides (K305. K307, K310) on or near the DIII lateral ridge, including AAs important for both binding of virus-neutralizing antibody and interacting with cellular receptors (Diamond et al., 2008; Sukupolvi-Petty et al., 2007).

Because GAGs are associated with the surfaces of many cultured mammalian cells, cell-specific flaviviral attachment – if

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0042-6822/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.virol.2013.03.011

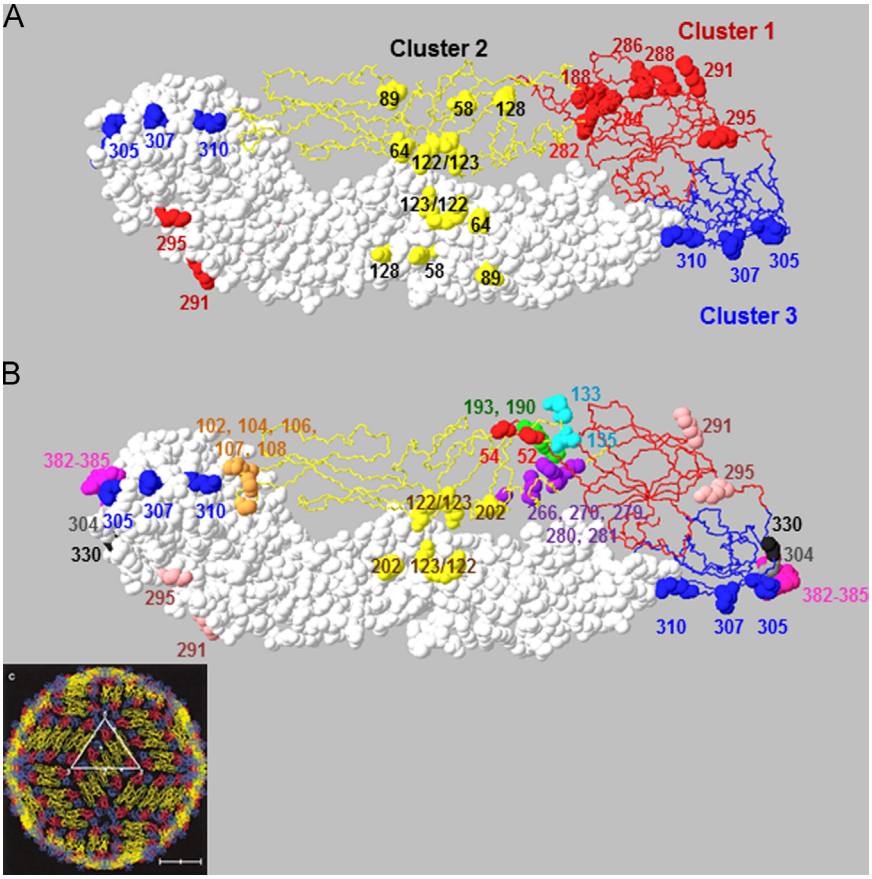

Fig. 1. Locations of HS-binding amino acid residues and mutations introduced in DENV2 E protein homodimers. Panel A: Location of possible HS-binding residues in the three potential binding clusters (Modis et al., 2003). Colors indicate locations in DI (red), DII (yellow) or DIII (blue). Panel B: backbone depiction of one monomer identifies locations of all mutations discussed. The DIII-FG (VEPG) loop mutants (dark pink), fusion peptide mutants (orange), and hinge region mutants (red, green, purple, and cyan) and have been previously described (Huang et al., 2010). Heparan sulfate-binding and DIII mutants (yellow, light pink, gray, and blue) and the G304K compensatory mutation G330D (black) described in this paper are also depicted. The second glycoprotein monomer is shown in a space-filling model to illustrate residue surface accessibility. The inset shows the configuration of homodimers in the surface of the virion (Kuhn et al., 2002). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

it exists – cannot be explained by interactions with only these AAs. Cell-specific interactions that result in differential DENV tissue-tropisms in vivo probably involve other plasma membrane receptors and potentially other E protein AAs. Several candidate cell membrane proteins have been shown to bind DENV in vitro but all are still poorly characterized (Martinez-Barragan and del Angel, 2001; Ramos-Castaneda et al., 1997). For example, it has been suggested that different cell surface proteins might be responsible for DENV binding to cultured monkey kidney cells (Vero) versus human hepatoma cells (HepG2) (Marianneau et al., 1996). Several size classes of mosquito cell (C6/36) membrane proteins appear to bind DENV, but these proteins also remain uncharacterized (Munoz et al., 1998; Salas-Benito and del Angel, 1997).

One surface protein, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), has been well-studied as a flavivirus attachment protein (Boonnak et al., 2008; Davis et al., 2006a, 2006b). DC-SIGN binds to virion proteins containing mannose glycans, and appears to be involved in DENV infection of primary DCs or DC-SIGN-expressing transformed cell lines—primarily through the carbohydrate moiety at the N67 glycosylation motif of the E protein (Pokidysheva et al., 2006). Since oligosaccharides on mosquito cell-derived DENVs also contain high mannose glycans, attachment to DCs via DC-SIGN may be an important first step following mosquito-bite transmission of virus to humans (Hacker et al., 2009; Hsieh and Robbins, 1984).

The region of the E protein that might bind to cell-specific receptors remains controversial, partly because the virion surface glycoprotein contains no well-defined spike structures common to other enveloped viruses (Kuhn et al., 2002; Mukhopadhyay et al., 2003, 2006; Pletnev et al., 2001; Zhang et al., 2002). DIII was first hypothesized to contain the primary receptor-binding motif because DIII-reactive neutralizing monoclonal antibodies (MAbs), and to a lesser extent DI-reactive MAbs were most effective in blocking DENV attachment to and infection of Vero cells (Crill and Roehrig, 2001). MAbs against DII epitopes had measurable, but lower, blocking activity. Several other reports also suggest that DIII binds cellular receptors (Gromowski and Barrett, 2007; Modis et al., 2003, 2005; Roehrig et al., 1998; Sukupolvi-Petty et al., 2007; Thullier et al., 2001; Volk et al., 2007). Several AA changes in DIII result in phenotypic changes usually associated with viral attachment, e.g., host range, tissue tropism, or virulence, and DIII also contains the most effective virus neutralization sites (Jennings et al., 1994; Modis et al., 2005; Rey et al., 1995; Roehrig, 2003; Roehrig et al., 1998). For WNV, DII also elicits effective neutralizing antibodies in humans (Vogt et al., 2009).

Hung et al., demonstrated that soluble heparin blocked binding of recombinant DIII (EIII) of DENV2 E-protein to BHK-21 cells, but not to C6/36 cells(Hung et al., 2004). They also demonstrated that a peptide representing AAs 380–389 of DIII (containing the DIII-FG loop) inhibited binding of EIII to C6/36 but not BHK21 cells. These results suggested that DENV binding to mammalian and mosquito cells might be through different receptors. However, we have previously determined that a mutant DENV2 with a deletion of the DIII-FG loop (382VEPG385) was capable of infecting C6/36 cells,