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A novel tetraspanin C189 upregulated in C6/36 mosquito cells following dengue 2 virus infection

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Abstract

Dengue (Den) viruses cause apoptosis in mammalian cells, but usually result in high progeny yields without evident damage in mosquito cells. By using subtractive hybridization, 13 potentially virus-induced genes were selected in Den-2 virus-infected *Aedes albopictus* C6/36 cells. Based on semi-quantitative and real-time RT-PCR, one novel gene, named C189, was significantly upregulated in infected C6/36 cells. Its full-length of 678 nucleotides (nt) was determined by a combination of 5′- and 3′-RACE products. After alignment, C189 was classified as a member of the tetraspanin superfamily that typically has 2 short cytoplasmic sequences, 4 transmembrane domains, as well as small and large extracellular regions (EC1 and EC2). It contains the hallmark CCG motif in the EC2 region and additional 17 conserved nucleotides as do other tetraspanins. C189 was not upregulated by inoculation of UV-inactivated Den-2 virus to C6/36 cells. This suggests that tetraspanin upregulation is not related to virus binding to the cell surface, and that C189 does not function as a receptor for dengue virus entry. On the other hand, overexpression of C189 was concurrent with viral proteins, targeting the plasma membrane of C6/36 cells infected with Den-2 virus. It is presumably beneficial or essential for cell-to-cell spread of the virus due to the role of tetraspanins demonstrated in intercellular adhesion.

Keywords: Cell–virus interaction; C189; Dengue virus; Tetraspanin

1. Introduction

Dengue virus is one of some 70 members of the family Flaviviridae and is transmitted between humans by mosquito vectors, mainly *Aedes aegypti* and *Aedes albopictus*. The genome of dengue viruses contains a positive-sense single-stranded RNA of approximately 11 kb in length (Chambers et al., 1990). In vitro culture of dengue viruses has been established in a number of mammalian and mosquito cell lines with rather-different cellular responses (Summers et al., 1989; Bonner and O'Sullivan, 1998). In mammalian cells, dengue virus infection begins after the virus binds to cell surface receptors, primarily heparan sulfate (HS) that is one type of GAG, and is internalized via the pathway of endocytosis (Chen et al., 1997). Endosomal acidification subsequently induces a conformational change in the envelope protein, resulting in membrane fusion and release of the viral nucleocapsid from the endosome (Heinz and Allison, 2001). A host signalase associated with endoplasmic reticula is known to be involved in co- and post-translational processing of the polyprotein encoded by viral RNA (Ruiz-Linares et al., 1989). After completion of replication, mature viral particles use the host secretory system to escape the cell (Heinz and Allison, 2000). In a word, host factors probably participate in all steps of virus infection, including entry, gene expression, virion assembly, and release (Ahlquist et al., 2003).

Inevitably, gene expression of host cells is modulated during virus infection (Ahlquist et al., 2003). Mammalian cells usually end up undergoing apoptotic cell death in response to dengue virus infection (Courageot et al., 2003). On the other hand, dengue virus does not cause deleterious effects in mosquito cells and may result in persistent infection, although the infected mosquito cells may form giant cells or syncytia (Chen et al., 1994). This reveals that the cellular response to virus infections is highly related to the cell type.

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Dengue virus infection of mammalian cells is generally dependent on interactions of the virus with glycosaminoglycan (GAG) molecules or other receptors on the cell surface (Bartosch et al., 2003), which may be linked to viral tropism and pathogenesis in the mammalian host (Summers et al., 1989). In mosquito cells, HS has been demonstrated sparsely distributed on the cell surface (W.J. Chen, unpublished data). As a result, it is worthwhile investigating in detail how dengue virus infects mosquito cells and how it spreads cell-to-cell once the infection has been established. A database recently established from expressed sequence tags (ESTs) of the mosquitoes *Armigeres subalbatus* and *Ae. aegypti* has provided advantages for screening genes that are regulated or modulated by dengue virus infection (Bartholomay et al., 2004).

Subtractive hybridization is a useful approach for sketching gene expression profiles that are activated during virus infection. A novel member of the tetraspanin superfamily, C189, was recently identified via this technique using cDNAs prepared from 2 populations of C6/36 cells, one infected by Den-2 virus and the other uninfected. The sequence of C189 was constructed through 5′- and 3′-rapid amplification of cDNA end (RACE) analysis. In order to understand the possible role of C189 during Den-2 virus infection, this study attempted to investigate the feature of C189 expression and its association with the virus infection in C6/36 cells.

2. Materials and methods

2.1. Cell culture and virus

Ae. albopictus C6/36 cells were grown in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) with non-essential amino acids and 10% fetal bovine serum (FBS) at 28 °C in a closed system of an incubator. The protocol for cell culture mostly followed the description reported previously (Chen et al., 2004). Cells for the test in this study were incubated for 24 h with Den-2 virus (New Guinea C) at a multiplicity of infection (MOI) of 0.1. Mock infection in the same cells was used for the control group throughout the study. Titration of Den-2 virus was carried out by plaque assay on BHK-21 cells as previously described (Chen et al., 2004). BHK-21 cells were cultured at 37 °C in an incubator with 5% CO_2.

2.2. UV-inactivation of Den-2 virus

Complete inactivation of the virus by UV radiation was performed at room temperature by exposure of a virus suspension to a low-intensity UV lamp (254 nm; 120 mJ/cm^2) for 30 min. The efficacy of virus inactivation was examined using a plaque assay. The expression of the C189 gene in C6/36 cells exposed to mock- or UV-inactivated Den-2 virus was assayed by real-time RT-PCR as described below.

2.3. Virus titration

A plaque assay was used to determine the virus titer following a method described previously (Chen et al., 2004). The virus titer was calculated based on the number of plaques formed in wells of the culture plate and was expressed as plaque forming units per milliliter (PFU/ml).

2.4. RNA extraction and cDNA synthesis

The procedures for RNA extraction and cDNA synthesis were described previously (Liu et al., 2004). In brief, total RNAs of both infected and control cells were extracted with the Trizol reagent (Invitrogen). cDNA was synthesized from the extracted total RNA using the SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA).

2.5. Subtractive hybridization

Subtractive hybridization was performed using 2 populations of synthesized cDNAs derived from C6/36 cells with the PCR-Select cDNA Subtraction Kit (Clontech), following the description in a previous report (González-Agüero et al., 2005). Briefly, cDNAs from C6/36 cells with Den-2 virus or mock infection were prepared in order to generate a subtracted cDNA library. As the virus infection enriched the cDNAs, the cDNA derived from Den-2 virus-infected cells was referred to as the tester, whereas that derived from the mock infection was the driver. Each type of cDNA was subjected to ligation in plasmid DNA after subtraction.

2.6. Screening of the subtracted cDNA library

The PCR products amplified from subtracted cDNA were cloned using the pGEM-T Vector (Promega, Madison, WI). Bacterial clones were isolated following transformation in order to create a subtracted cDNA library that was subsequently hybridized with digoxigenine (DIG)-labeled DNA probes derived from either virus infection or mock infection as the method described previously (González-Agüero et al., 2005). The anti-DIG antibody conjugated with alkaline phosphatase (Roche, Germany) and the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) colorimetric substrate was then used to visualize all dots on each membrane.

2.7. Semi-quantitative reverse-transcriptase polymerase chain reaction (SQ-RT-PCR)

Primers designed from the partial sequence of the selected upregulated gene were used to run RT and the subsequent PCR cycles (Table 1). A portion of the DNA products was removed from each sample at 10, 20, 30, and 40 cycles during thermocycling at PCR conditions described previously (Chen et al., 2004). The PCR products were then analyzed by electrophoresis on 2% agarose gels.

2.8. Real-time RT-PCR

Real-time RT-PCR was performed with SYBR Green PCR Master Mix using primers for the semi-quantitative RT-PCR on an ABI prism 7000 Sequence Detection System (Applied