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Interferon-mediated ISG15 conjugation restricts dengue virus 2 replication

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ABSTRACT

ISGylation, an ubiquitin-like post-translational modification by ISG15, has been reported to participate in the interferon (IFN)-mediated antiviral response. In this study, we analyzed the functional role of ISGylation in dengue virus 2 (DENV-2) replication. Overexpression of ISG15 was found to significantly suppress the amount of extracellular infectious virus released, while intracellular viral RNA was unaffected. This effect was not observed with a conjugation-defective ISG15 mutant. In addition, extracellular virus infectivity was decreased by ISG15 overexpression. To further clarify the role of ISGylation in the anti-DENV-2 response, we depleted endogenous ISG15 by RNA interference and analyzed the virus production in the absence or presence of type-I IFN. Results showed a significant reduction in extracellular DENV-2 RNA levels for cells treated with IFN, and that these DENV-2 RNA levels could be partially restored by the ISG15 knockdown. Among various DENV-2 proteins, NS3 and NS5 were subjected to the ISGylation. These results demonstrate that IFN-inducible ISGylation suppresses DENV-2 particle release, and that ISG15 is one of the mediators of IFN-induced inhibition of DENV-2 replication. ISG15 therefore functions as a host antiviral factor against DENV-2 infection.

1. Introduction

Dengue is the most common arthropod-borne viral infection in the world. Dengue virus (DENV) is found in more than 100 countries and is still spreading to new areas every year [1]. The presence of four antigenically distinct serotypes of DENV (DENV-1 to 4) is important with regard to the clinical manifestations of dengue. Primary infection with one serotype is often asymptomatic or manifests as self-limiting dengue fever (DF), but antibodies produced during this primary infection are non- or sub-neutralizing to heterologous serotypes and can enhance secondary infection, resulting in more severe and life-threatening manifestations such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2].

DENV is an enveloped, single sense positive-strand RNA virus belonging to the *Flaviviridae* family. The viral RNA is translated at the endoplasmic reticulum to produce a polyprotein, which is further processed by cellular and viral proteases into three structural proteins (C, prM and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The structural proteins are assembled into the virus particle, while the NS proteins are responsible for the replication of the viral RNA genome [3].

The primary targets of virus infection in humans are the monocyte lineage cells, including dendritic cells and macrophages [4]. Although these cells play an important role in the innate and protective immunity against virus infections, the mechanisms of immunopathogenesis and the host immune response in DENV infection remain largely unknown. Since no approved vaccines or antiviral drugs are available for the prevention or treatment of dengue, understanding the immune response during DENV infections will contribute to the development of DENV therapeutics.

Induction of the interferon (IFN) response is central to host protective immunity and its signaling pathways have an essential function in the triggering of innate immune responses against pathogens [5]. DENV infection is a weak inducer of the IFN

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responses and can block IFN signaling pathways to some extent [6], but treatment with type-I and type-II IFNs can limit virus replication *in vitro* during the initial stage of DENV infection [7]. In addition, it has been reported that DF patients have higher levels of circulating IFN-α and IFN-γ as compared to DHF/DSS patients, suggesting that IFN levels are one of the determinants of clinical outcome in DENV infection [8]. Furthermore, DENV is only able to replicate in mice deficient in IFN receptors [9] or an IFN signaling component, STAT2 [10]. These studies indicate that the IFN system is part of the host protective immune response that controls DENV replication and pathogenesis.

It is well known that the anti-viral effects of IFN are mediated by IFN-stimulated genes (ISGs), some of which are also induced by virus infection [11]. Several ISGs have also been reported to inhibit distinct steps of the DENV replication cycle, though the suppression mechanisms remain unclear [12,13]. ISG15 is a type-I IFN inducible, 17 kDa protein that contains two ubiquitin-like domains. Like ubiquitin, ISG15 exists as a free molecule and can be conjugated to other proteins. In human cells, the ISG15 conjugation (ISGylation) is catalyzed by Ube1L (E1 enzyme), UbcH8 (E2 enzyme), and EFP/HERC5 (E3 enzymes), which are also induced by type-I IFN. Importantly, ISGylation has been demonstrated to cause either a gain or loss of function in target proteins [14].

Negative regulation of viral replication by ISGylation has been reported in human immunodeficiency virus, influenza virus and hepatitis C virus [15–17]. With respect to flaviviruses, a recent study using a mouse macrophage cell line showed that the depletion of ISG15 by small interference RNA (siRNA) increased DENV and West Nile virus (WNV) replication [18]. However, the detailed mechanism underlying the anti-DENV effect of ISG15 remains unclear. In the present study, we report that ISG15 is one of the mediators of type-I IFN-induced inhibition of DENV replication in human cells, and that ISGylation was indispensable for the ISG15-mediated anti-DENV effect, which was likely to suppress viral particle release.

2. Materials and methods

2.1. Cells and viruses

Human cervical cancer cells (HeLa), and human embryonic kidney cells (HEK293T) were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 μg/ml penicillin and 100 μg/ml streptomycin sulfate. Baby hamster kidney cells (BHK-21) were grown in Roswell Park Memorial Institute 1640 medium (RPMI, Life Technologies) supplemented with 10% FBS and antibiotics. All cells were incubated at 37 °C with 5% CO_2. *Aedes albopictus* mosquito cells (C6/36) were maintained at 28 °C in HEPES-modified RPMI supplemented with 10% FBS and antibiotics.

High-titer stock of DENV-2 was prepared by inoculating C6/36 cells with DENV-2, which had been isolated in Singapore [19], at a multiplicity of infection (MOI) of 0.1 and cultured for 5 days. The supernatants were collected and centrifuged at 1000 × *g* for 5 min. Infectious viral titer was determined by plaque assays using BHK-21 cells as described below.

2.2. Plaque assay

BHK-21 cells were seeded in 24-well plates at 1.5×10^5 cells/well. Serial dilutions of virus supernatants were added and incubated at 37 °C for 1 h, followed by addition of RPMI containing 2% FBS, antibiotics and 0.8% carboxymethylcellulose (CalBiochem) to each well. After 5 days of incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 20 min. The plaques were visualized by staining with 1% crystal violet. Virus titer was calculated as plaque-forming units (PFU) /ml.

2.3. Plasmid DNA

cDNAs of DENV structural and NS proteins were amplified either from pXJ-C-prM-E (encoding structural genes [20]) or pWSK-DENV-Rep (encoding NS genes [20]) by PCR and cloned into the *Not*I-*Xho*I site of pCAG vector containing N- (for C, NS2B and NS3 expressions) or C-terminal FLAG (for prM, NS1, NS4A, NS4B and NS5 expressions) sequence. The expression plasmids of human ISG15 (ISG15-pCAG-HA), Ube1L (Ube1L-pcDNA3) and Ubch8 (Ubch8-pcDNA3) have been described previously [21]. Site-directed mutagenesis of the ISG15 gene was carried out by inverse PCR method [22] (ISG15-AA-pCAG-HA).

2.4. Intracellular and extracellular DENV infectivity assay

The DENV infectivity assay was performed as described previously [23] with slight modifications. HeLa cells (4×10^5 cells in a 60 mm dish at 1 day prior transfection) were transfected with 5 μg of ISG15-pCAG-HA, 1.5 μg of Ube1L-pcDNA3 and 1.5 μg of Ubch8-pcDNA3. Four hours after transfection, cells were inoculated with DENV-2 at MOI of 10 for 2 h. Inoculated cells were washed three times with PBS, and then cultured for 36 h. Infected cells were washed twice with PBS, collected by centrifugation, and suspended in distilled water. Cells were then sheared by 10 strokes with a 22-gauge needle before incubation at room temperature for 15 min. The lysate supernatant was collected after centrifugation at 15,000 × *g* for 5 min, filtered through a 0.45-μm filter (Millipore), and concentrated with a Vivaspin-6 centrifugal filter (GE Healthcare). The solvent was exchanged with DMEM, and the resulting solution was used for the intracellular DENV quantification. Culture supernatant at 36 h after infection was used to quantify the extracellular virus titer. Virus titer in each sample was analyzed by plaque assay as described above.

2.5. RNA interference experiment

A siRNA duplex targeting human ISG15 (si-ISG15: 5′-UGAGCAC CGUGUUCAUGAA-3′) and a negative control siRNA duplex (si-control) were purchased from Integrated DNA Technologies. HeLa cells (2×10^4 cells/well at 1 day prior transfection), were transfected with 50 nM si-ISG15 or si-control using Silentfect (Bio-Rad). Four hours after transfection, cells were treated with 1000 units/ml type-I IFN (a mixture of human interferon α and ω, Sigma) for 8 h, and then inoculated with DENV-2 at a MOI of 1. Intracellular and extracellular DENV RNA were extracted 36 h after infection and quantified by real-time RT-PCR analysis (see below). Expression levels of ISG15 were analyzed by immunoblotting at 24 h after IFN treatment.

2.6. Immunoblotting analysis

Protein samples were separated by 7% SDS–PAGE gel and transferred to Immobilion P transfer membrane (Millipore). The primary antibodies used were anti-HA rabbit monoclonal (C29F4, Cell Signaling), anti-FLAG mouse monoclonal (M2, Sigma), anti-actin mouse monoclonal (AC40, Sigma), and anti-ISG15 rabbit polyclonal (2743, Cell Signaling) antibodies. Horseradish peroxidase-linked anti-mouse IgG or anti-rabbit IgG (Cell Signaling) was used as a secondary antibody. Proteins were detected using an ImageQuant LAS4000 mini chemiluminescent image analyzer (GE Healthcare).