Chapter 1

Cellular Control of Dengue Virus Replication: Role of Interferon-Inducible Genes

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Additional information is available at the end of the chapter

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Abstract

Dengue, one of the most common mosquito-borne viral infectious diseases in the world, is caused by the dengue virus (DENV). This enveloped RNA virus has immunologically distinct serotypes that increase the risk of life-threatening diseases, such as dengue haemorrhagic fever. However, no effective antiviral therapy against DENV infection has yet been established. As seen in other RNA viruses, various cellular factors have been reported to participate in efficient DENV replication. On the other hand, increasing recent evidence demonstrates that host cells harbour inhibitory factors that limit the DENV replication. In particular, it is well known that the response of interferons (IFNs), the first line of a host defence system against invading pathogens, evokes the expression of a number of genes that negatively regulate various steps of virus replication. This set of inhibitory genes, called interferon-stimulated genes (ISGs), is considered to be a central force in IFN-mediated antiviral responses. In this chapter, we focus our attention on the cellular factors involved in DENV infection, particularly to those that modulate DENV replication through their association with viral RNA. In addition, we also summarize general experimental approaches for identifying the host factors of RNA viruses, including DENV.

Keywords: dengue virus, cellular factors, RNA untranslated regions, interferon-stimulated genes, identification systems

1. Introduction

Dengue virus (DENV) is an enveloped and positive-strand RNA virus that belongs to the genus *Flavivirus* of the Flaviviridae family [1]. An important characteristic of the Flavivirus is that this genus consists of a large number of arthropod-borne viruses, many of which are transmitted by mosquitoes and ticks. In addition, flavivirus infection often causes

life-threatening diseases in humans, such as haemorrhagic fever, encephalitis, and meningitis [2]. Recently, the Zika virus (ZIKV), a member of the flavivirus family that has spread explosively throughout the Americas, is reported to be associated with neurological complications [3, 4]. Flaviviruses, therefore, have significant clinical as well as economic impacts on modern society.

DENV is a mosquito-borne virus widely distributed in the tropical and subtropical areas of the world. This flavivirus infection is transmitted to humans via the bite of infected mosquitos. The primary vector of DENV infection is *Aedes aegypti*, while *Aedes albopictus*, which originated in Asia but has extended its range to other regions of the world, is also capable of spreading a dengue outbreak. DENV has four antigenically distinct serotypes (from DENV-1 to DENV-4). Primary infection with one of the serotypes is often asymptomatic or causes self-limiting dengue fever (DF). However, secondary infection of different serotypes increases the risk of more serious forms of DENV infection, such as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), due to the presence of non- or sub-neutralizing antibodies generated during the primary infection. Therefore, dengue is a significant threat to humans, yet there is currently no specific antiviral available for DENV infection [1, 5]. However, it should be noted that a live attenuated vaccine against DENV developed by Sanofi Pasteur (Dengvaxia) has been licensed for use in a limited number of countries, including Mexico and the Philippines, although the efficacy of the DENV vaccine in endemic countries is still under investigation [6].

2. Brief overview of DENV replication

DENV infection begins with its entry into a permissive cell via receptor-mediated endocytosis (**Figure 1**). So far, various types of human cells, such as macrophages, lymphocytes, hepatocytes, and endothelial cells, are reportedly susceptible to DENV infection. Among them, monocyte lineage cells (i.e., dendritic cells [DCs] and macrophages) are thought to be the primary targets of DENV in humans. As the entry receptors of DENV, several cellular proteins, including C-type lectin receptors (e.g., DC-SIGN/CD209, mannose receptor/CD206) and phosphatidylserine receptors (e.g., TIM, TAM), have been demonstrated [7].

Upon entry into the cell, a membrane fusion between DENV envelope (E) glycoprotein and endosomal vesicle occurs, leading to the release of viral RNA into the cytoplasm. The DENV genome is a single-stranded positive-sense RNA and is approximately 10-kb long. The viral RNA contains a single long open reading frame (ORF) flanked by highly structured 5′ and 3′ untranslated regions (UTRs), with a type 1 cap (m7GpppAmp) at the 5′ terminus and no poly(A) tract at the 3′ terminus [8]. The single ORF is translated to a large polyprotein, which is subsequently cleaved co- and post-translationally into three structural (capsid [C], pre-membrane [prM], and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by viral (NS3) and host proteases (**Figure 1A**). Of the NS proteins, NS5, the largest viral protein, functions as an RNA-dependent RNA polymerase (RdRp), which synthesizes a complementary minus-strand RNA template and, in turn, produces many copies of

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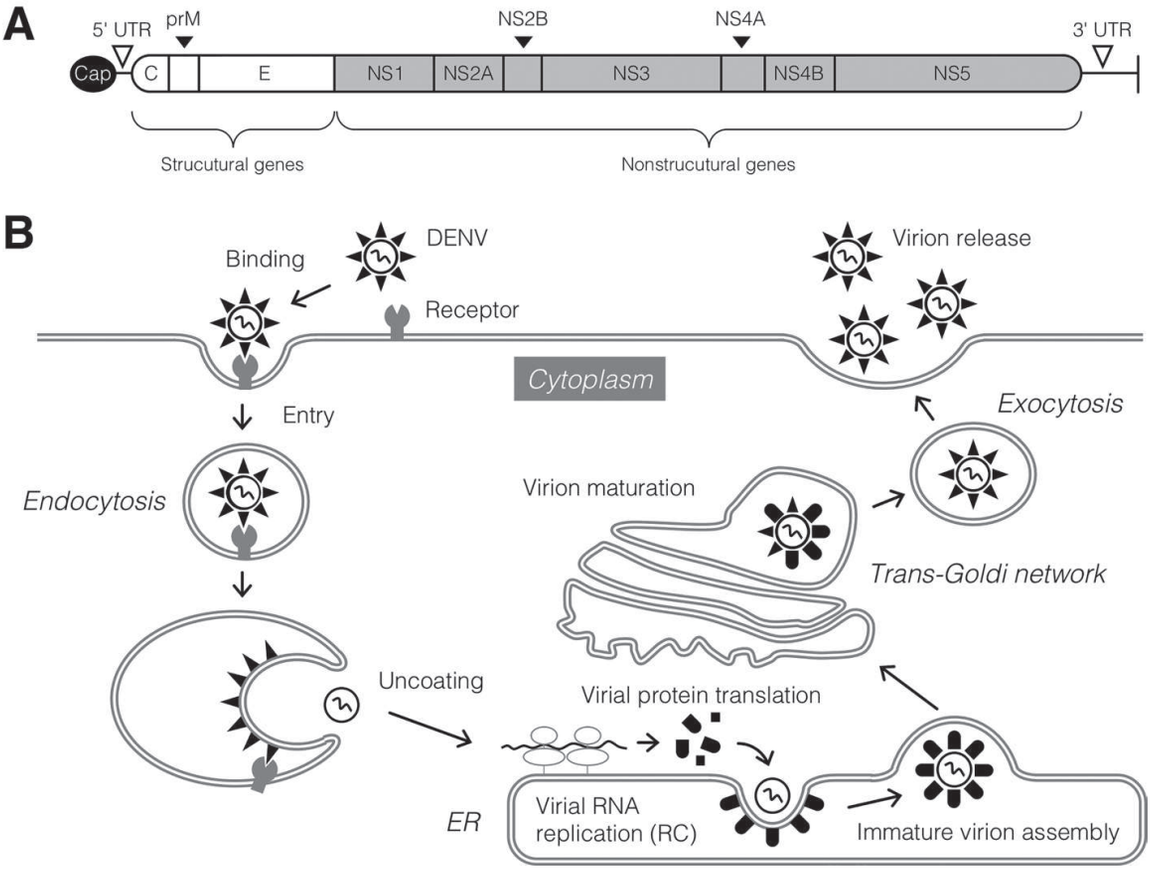

Figure 1. The genome structure and replication cycle of DENV.

positive-strand viral genomic RNA. Viral RNA replication takes place in the so-called replication complexes (RCs) composed of viral RNA and proteins as well as hypothetical cellular proteins, which are formed on the endoplasmic reticulum (ER) membrane. This membrane structure, rearranged by DENV infection, is also the place of viral RNA translation, protein processing, and progeny virion assembly [9]. The immature particles then bud into the ER lumen and are transported through the secretion pathway of the trans-Golgi network, in which progeny virions undergo a maturation process via a conformational change of prM and E proteins on the virion surface. Finally, mature, infectious virions egress from infected cells by exocytosis (**Figure 1B**) [5, 8].

3. Cellular factors involved in DENV replication: interaction with viral RNA untranslated regions

As seen in other RNA and DNA viruses, flaviviruses, including DENV, have been shown to utilize biological processes of the host to replicate efficiently in infected cells through the interaction between viral and cellular proteins. Although the biological relevance of many of the host interactors in *in vivo* replication and the pathogenicity of DENV remain unclear,

those virus-host interactions could serve as attractive targets of antiviral drugs [2]. In addition to the entry receptors, intracellular factors implicated in the replication cycle of DENV have been comprehensively reviewed elsewhere [2, 8, 10, 11]. Meanwhile, it is becoming apparent that various cellular cofactors are recruited into the 5′ and 3′ UTRs of DENV RNA, which results in enhanced virus replication [12].

The DENV 5′ and 3′ UTRs are approximately 100 and 350–700 nucleotides long, respectively (**Figure 1A**). The primary sequences located within or adjacent to the UTRs have been shown to be essential for virus replication [8]. For instance, complementary sequences, termed CS (cyclization sequence) elements, found in the ORF of capsid and the 3′ UTR and UAR (upstream of the AUG region) elements, found in the 5′ and 3′ UTRs, are reported to physically anneal to mediate DENV genome cyclization. This cyclization is likely to recruit the DENV RdRp at the 5′ end of the viral genome and then likely to facilitate viral RNA amplification [13]. Additionally, DENV UTRs form stable secondary and tertiary structures, and these structural integrities are thought to be important for the regulation of viral RNA synthesis and translation process. Therefore, albeit the sequences are diverse, UTR secondary structures are highly conserved among flaviviruses [8]. Furthermore, these regions have been demonstrated to contain several cellular proteins [12].

An early study that employed an *in vitro*-formed nucleoprotein complex of synthesized DENV 3′ UTR and mosquito cell extracts identified some RNA-binding proteins—the La autoantigen, translation elongation factor-1α (EF-1α), and polypyrimidine tract-binding protein (PTB)—as the DENV UTR-associated cellular factors in cells [14]. Subsequent studies have also revealed that human La protein binds not only with the DENV 3′ UTR but also with the 5′ UTR [15, 16]. Interestingly, interactions of La protein with viral proteins NS3 and NS5 were shown, suggesting that the La protein is somehow involved in the function of RC in infected cells [15]. The La autoantigen is reported to associate with RNA polymerase III transcription; interestingly, this RNA-binding protein has also been shown to stimulate the translation of viral and cellular mRNAs by binding with their UTRs [17]. As with mosquito EF-1α, its human homologue (i.e., EF1A) has been reported to specifically recognize the conserved 3′-terminal stem-loop (SL) in the 3′ UTR of West Nile virus (WNV) RNA [18]. Given the colocalization of EF-1α with the DENV RC in the infected cell [19], it can be suggested that this cellular protein also plays an important role in the function of flaviviral RNA. However, the precise step of flaviviral replication in which EF-1α is involved remains to be elucidated [19].

PTB is a ubiquitous RNA-binding protein known to be involved in splicing, polyadenylation, stability, and translation of cellular mRNA [20]. With regard to its role in virus infection, PTB has been shown to bind to the UTRs of picornaviruses [21, 22] and the hepatitis C virus (HCV) [23]; it functions as an internal ribosome entry site, (IRES)-trans-acting factor, to activate viral translation [24, 25]. On the other hand, several studies have raised questions about the involvement of PTB in the translation process of those RNA viruses [26–28]. Interestingly, both La and PTB were implicated in HCV replication [29]. In the case of DENV infection, PTB is reportedly required for efficient replication. Furthermore, PTB interacted with a DENV protein, NS4, as well as viral RNA, suggesting that PTB associates with DENV RCs [30]. Although it remains unclear whether PTB regulates DENV RNA simplification or

the translation process (or another step of virus infection), PTB may function as a molecular chaperone to stabilize the structured viral RNA [30, 31]. In addition to the host factors mentioned above, recent studies using tobramycin RNA aptamer affinity chromatography identified DDX6 (DEAD-box RNA helicase) and ERI3 (putative 3′–5′ RNA exonuclease) as DENV UTR-binding cellular proteins that promote DENV replication [32, 33].

The genome of flaviviruses, including DENV, encodes enzymes required for viral RNA synthesis (i.e., RdRp and helicase) and viral protein processing (i.e., protease); however, the viral protein translation process must rely fully on the translational machinery of the host [34], except for the methyltransferase activity conferred by NS5, which adds a type 1 7-methylguanosine cap to the 5′ terminus of viral RNA. It is, therefore, not surprising that cellular factors are associated with DENV RNA during viral translation [8, 35]. At the initiation step of eukaryotic mRNA translation, a 5′ cap structure is first recognized by a eukaryotic initiation factor, eIF4E, which, in turn, leads to the recruitment of eIF4G. eIF4G serves as a scaffold protein that binds the DEAD-box RNA helicase eIF4A and also the poly(A)-binding protein (PABP), resulting in the circularization of mRNA [8]. This complex formation induces the association of the 43S ribosomal subunit through the binding of the eIF3 complex. Finally, the 60S ribosomal subunit joins the initiation complex that enables the elongation process of translation [8]. In the case of DENV, its genomic RNA is 5′ capped but lacks 3′-end poly(A) sequences [8]. Nevertheless, PABP is shown to interact with DENV 3′ UTR *in vitro* by recognizing the A-rich regions upstream of 3′ SL in the 3′ UTR [36]. Indeed, a study using chimeras reported that mRNA based on a cellular (globin) gene and DENV RNA revealed that DENV 3′ UTR exhibits functions similar to those of polyadenylated non-viral UTRs in enhancing translation rather than RNA stabilization [37]. Thus, it is plausible that as with cellular mRNA, translated DENV RNA also forms a closed-loop structure via association with host translation factors [38]. In addition, it is demonstrated that DENV RNA may be able to produce proteins by a 5′ cap-independent translation mechanism in certain cellular situations in which the eIF4E is starved and, thereby, canonical host translation is inhibited [39].

4. Experimental procedures for identifying the cellular factors

In the following sections, we summarize several general approaches to search for the cellular factors involved in virus infections.

4.1. Conventional methods using living cells

4.1.1. Gene expression analysis and proteomic analysis

Studies using a microarray system, quantitative RT-PCR analysis, and GeneChip analysis have revealed that the expression levels of many mRNAs in host cells are dramatically altered upon DENV infection [40–42]. It is highly possible that some of these DENV-responsible genes and their products function as crucial positive or negative regulators of DENV replication in cells. Indeed, tumour necrosis factor–related apoptosis-inducing ligand (TRAIL),

whose mRNA expression level significantly increased with DENV infection, was found to be a negative regulator of DENV replication [42]. In addition to the analysis of the gene expression profile, the global host protein expression profile upon DENV infection was also investigated by comprehensive proteomic analysis. Conventionally, the total host proteins extracted from infected cells and control cells were separated by two-dimensional PAGE (2-D PAGE), and the proteins whose expression levels were altered by the viral infection were picked up, and their amino acid sequences were determined with mass spectrometry (MS) analysis. Previous studies have identified many host factors that respond to DENV infection [43, 44]. More recently, a proteomic analysis based on stable isotope labelling by amino acids in cell culture (SILAC), which overcame the limitations of sensitivity and resolution of 2-D PAGE, was developed and applied to DENV research [45, 46]. The technologies of high-throughput gene expression analysis and proteomic analysis are thought to be powerful tools for understanding the global cellular expression profile of host cells upon DENV infection, both at the gene and at the protein levels. From the results of these assays alone, however, it is difficult to distinguish whether DENV-responsible host factors that are identified are indeed involved in the regulation of viral replication. To understand the roles and functions of these hit factors requires further functional analysis.

4.1.2. RNAi screening

RNA interference (RNAi) is a well-known approach for identifying novel host factors in virus-infected cells. When the knockdown of a host gene by RNAi alters the efficiency of viral replication, it is highly possible that this gene works as a host factor of the virus. Currently, commercial siRNA pools that cover most human genes are available, and many genome-wide comprehensive screenings for many kinds of viruses have been performed thus far [47–49]. In studies of flaviviruses, hundreds of host factor proteins involved with the early steps of WNV infection have been identified by silencing more than 20,000 human genes from a small interfering RNA (siRNA) pool [50]. An additional bioinformatic study, followed by the siRNA screening, revealed that many of these hits were involved in the ubiquitin-proteasome pathway and the ER-associated degradation (ERAD) pathway, both of which are essential for many steps of viral replication. In addition to host factors in human cells, one study aimed to identify host factors from insect cells, another host of DENV [51]. In that study of DENV-adapted cells from *Drosophila melanogaster* and an siRNA pool targeting more than 20,000 genes of *Drosophila*, the proteins that accelerate or inhibit the replication of DENV in *Drosophila* cells were identified [51]. Then human analogues of hit genes in *Drosophila* cells were subsequently silenced in DENV-infected human cultured cells, and 42 of these were found to be common host factors in human and *Drosophila* cells [51]. It is noteworthy that RNAi screening can identify host factors that can affect viral replication either by direct interaction with viral components or by indirect interaction, such as the regulation of the IFN pathway.

4.1.3. Yeast two-hybrid analysis

For researchers attempting to identify host factors that directly interact with viral proteins, yeast two-hybrid (Y2H) analysis is a common and effective way; numerous host factors of

many viruses, including DENV, have been identified using this system [52–58]. Taking advantage of good throughput, several large screenings were performed to build DENV host interactomes using human cDNA libraries. One study reported that a Y2H analysis using either partial or full-length DNA fragments encoding each DENV protein and the human liver cDNA library identified 105 viral-host interactions; further knockdown experiments using siRNAs revealed that six proteins were essential for the efficient viral replication of DENV [59]. Another study also carried out a Y2H assay using NS3 and NS5 proteins from DENV and other flaviviruses as bait, and 108 human proteins were identified as interacting with NS3 or NS5 or both [60]. In addition, Y2H assays were performed to identify DENV proteins interacting with host factors from human and mosquito cDNA libraries. They identified several common host factors conserved in both humans and mosquitos [61]. These studies identified many host proteins that interact with DENV proteins; however, few of the hits overlap in independent studies. Although this might be caused mainly by the fact that the assay in each study was performed in different conditions and with different cDNA libraries, it is also possible that each study includes many false positives and false negatives.

4.2. Wheat germ–based protein array system

4.2.1. Overview of the protein array technology

As described above, the living cell-based methods are powerful tools for identifying viral-host interactions since the assays could be carried out under physiological conditions, at least partially. However, these methods have several disadvantages. First, proteins whose expression levels are quite low or that show cytotoxicity are hard to analyse. Second, immunoprecipitation assays are commonly used to detect the interaction between a protein and a protein; however, the number of interactions that can be detected at one time is limited. Therefore, it is highly possible that many researchers have potentially overlooked important but difficult-to-detect interactions in their first screenings using living cells. To solve these problems, we recently developed a novel biochemical screening method based on a wheat germ cell–free protein synthesis system (wheat cell-free system) and high-throughput binding assay. The wheat cell-free system enables the synthesis of various kinds of eukaryotic proteins in a 96-well format [62]. So far, proteins having several transmembrane domains, relatively large molecular weights, and cytotoxic activity were successfully synthesized by this system [63, 64]. This robust protein synthesis system allows us to establish a "protein array", from which tens to thousands of recombinant proteins sorted by their functions were arrayed into each well of a 96-well plate [65]. The protein arrays currently available in our research group are shown in **Table 1** [65–70]. Based on these protein arrays, we have established a high-throughput binding assay for identifying proteins directly bound to target proteins from the array. To perform hundreds to tens of thousands of binding reactions, a luminescent-based binding assay, called AlphaScreen, was employed [65, 66, 71]. This assay is able to use crude translation products of wheat cell-free synthesis to detect binding reactions by mixing these crude proteins in a 384-well plate, followed by adding two beads and the antibody for detection. Because of its flexibility, AlphaScreen can be used not only as a binding assay but also as a protein cleavage assay for a viral protease. The principle of the assays is shown in **Figure 2**.

Protein array	Origin	Number of proteins	References
Protein kinase	Human and mouse	400	Masaki et al. [66], Miyakawa et al. [67], Kudoh et al. [68]
E3 ligase	Human and mouse	250	Takahashi et al. [65], Tan et al. [69]
Deubiquitinating enzyme	Human	85	Unpublished
Single transmembrane protein	Human and mouse	730	Unpublished
Auto-antigen protein	Human and mouse	2100	Matsuoka et al. [70]

Table 1. Wheat cell–free-based protein arrays currently available in our research group.

4.2.2. Practical applications for viral research

We and other research groups have done several assays using this technology to identify host proteins that interacted with viral proteins. In researching the HCV (a virus related to DENV), several protein kinases were found to bind directly with the HCV non-structural protein NS5A using the protein kinase array; by phosphorylating NS5A through additional functional analysis using a cultured cell system, one protein kinase, Casein kinase I-α, was found to regulate viral replication [66]. The protein kinase array was used to identify other host factors that

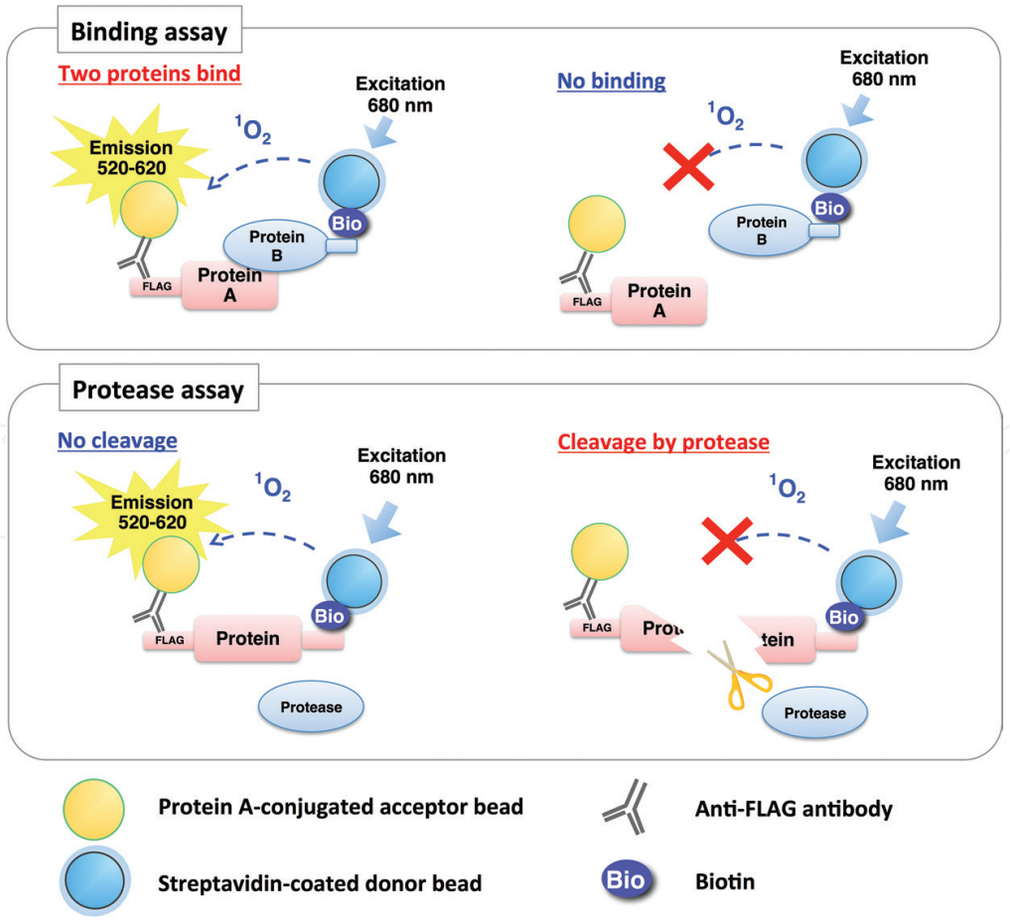

Figure 2. A schematic diagram of binding assay and protease assay based on AlphaScreen technology.

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functionally interacted with human immunodeficiency virus (HIV) proteins [67, 68]. In the case of the protease assay, one research article demonstrated that a protease from xenotropic murine leukaemia virus-related virus (XMRV) and 24 cellular proteins that were target candidates of the viral protease were synthesized; *in vitro* cleavage assay revealed several novel substrates of XMRV protease [72], indicating the feasibility of using wheat cell–free-based protein array technology and high-throughput biochemical assay based on AlphaScreen.

4.2.3. Wheat cell–free protein array for DENV research

Thus far, NS3 and NS5 proteins of DENV have been considered difficult to synthesize as full-length active recombinant proteins using a conventional protein expression system, such as for *Escherichia coli*. We previously reported that the wheat cell-free system successfully synthesized full-length NS3 and NS5 proteins in a soluble form; some biochemical analyses revealed that both recombinant proteins possessed enzymatic activities [63], indicating the usefulness of the expression system for the preparation of DENV proteins. In addition to these soluble proteins, NS4B, a protein with at least three transmembrane domains, was synthesized with the wheat cell-free system (**Figure 3A**). Our preliminary study demonstrated that NS4B was

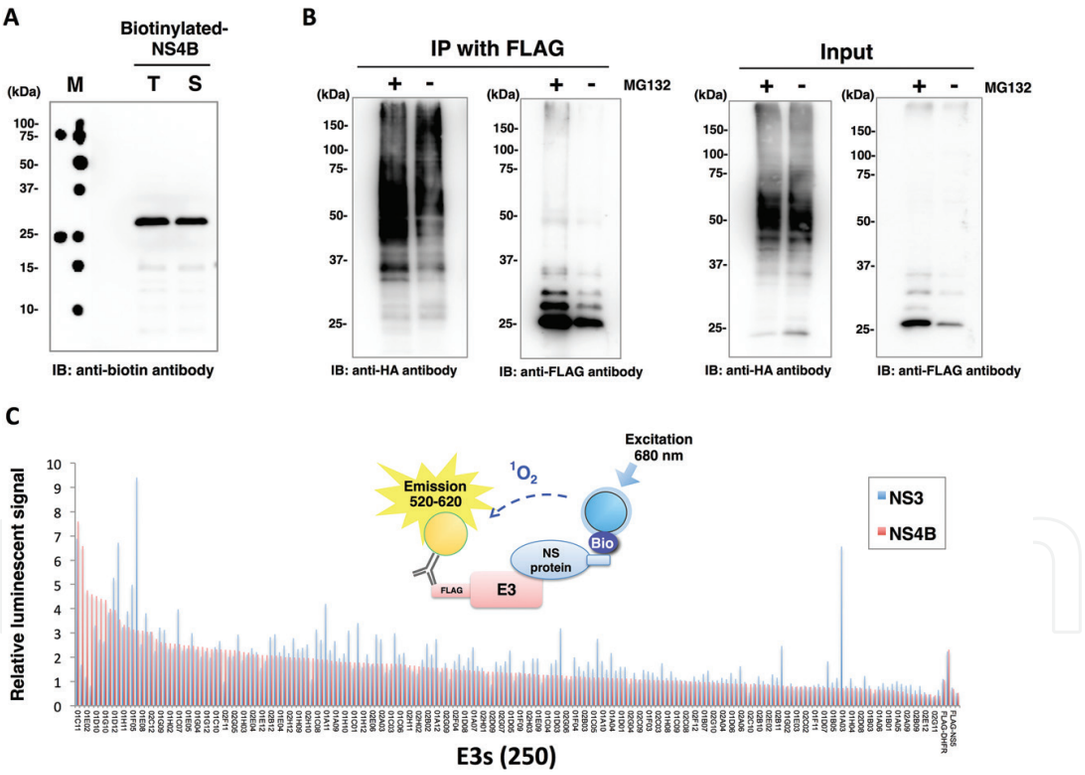

Figure 3. Identification of E3 ligases targeting DENV NS4B by wheat cell-free-based protein array system. (A) Expression of biotinylated NS4B with wheat cell-free system. The total translation products of NS4B (T) and supernatant (S) after centrifugation of the total translation product were subjected to SDS-PAGE, followed by immunoblot analysis using anti-biotin antibody. M is the molecular marker. (B) Ubiquitination of NS4B. HEK293T cells overexpressing FLAG-tagged NS4B and HA-tagged ubiquitin were treated with proteasomal inhibitor MG132, and FLAG-tagged NS4B was precipitated with anti-FLAG antibody. The NS4B and ubiquitin were detected by immunoblot analysis using anti-FLAG antibody and anti-HA antibody, respectively. (C) AlphaScreen assay to identify the E3s targeting DENV NS3 and NS4B proteins using the E3 protein array. Biotinylated NS3 and NS4B were used as bait, and biotinylated DHFR was used as negative control of NS proteins. The relative luminescent signal was calculated as follows: the value from E3 and NS4B/value from E3 and DHFR.

highly ubiquitinated when NS4B was overexpressed in HEK293T cells (**Figure 3B**), suggesting that the amount of NS4B expressed was regulated in host cells in a ubiquitin/proteasome-dependent manner. When a protein is ubiquitinated and, subsequently, degraded by the 26S proteasome, E3 is a determinant of the ubiquitination, as E3 specifically binds to the target protein and transfers activated ubiquitin from the ubiquitin conjugation enzyme, E2 [73]. Therefore, we screened NS4B-binding E3s from the E3 protein array using AlphaScreen, as we recently reported [65]. NS3 was used as control to determine the NS4B-specific E3s. As shown in **Figure 3C**, many E3s were found to bind with recombinant NS4B. Currently, additional functional analysis, such as *in vitro* ubiquitination assay and protein degradation assay in cells, is ongoing.

5. Restriction of DENV infection by cellular inhibitors

As previously mentioned, DENV hijacks the host's biological process for its efficient replication. Meanwhile, it has become apparent that DENV infection can be limited by cellular factors. In this sense, the innate immune response induced by IFN is considered to be the first line of defence against DENV [8]

Generally, RNA viruses that infect target cells are sensed by the pattern recognition receptors (PRRs), which specifically recognize a component of invading viruses. As for DENV infection, the membrane-bound Toll-like receptors (TLR3, TLR7, TLR8) and the cytosolic receptors (retinoic acid-inducible gene I [RIG-I], melanoma differentiation-associated gene 5 [MDA5]) are reported to be the PRRs for viral RNA [74–77]. These recognitions in turn activate adaptor molecules of the PRR, leading to the activation of a downstream phosphorylation cascade and the subsequent production of IFN and pro-inflammatory cytokines (**Figure 4**) [78]. Among the IFNs produced, type I IFNs, including IFNα, IFN-β, and IFN-ω, play an important role in antiviral immunity [79]. The type I IFN then binds to its receptors (IFNAR) on neighbouring cells and signals to induce the phosphorylation of signal transducers and the activators of transcriptions 1 and 2 (STAT1 and 2) in cytoplasm. This phosphorylation of STAT1/2 triggers the formation of IFN-stimulated gene factor 3 (ISGF3) with IFN-regulatory factor 9 (IRF9). Finally, the ISGF3 complex translocates to the nucleus and acts as a transcription activator for the expression of a number of genes by binding to the IFN-stimulated response elements (ISREs) on chromosomes (**Figure 4**) [80].

As seen above, IFN is considered to be an inducer of the antiviral state, and it has been well demonstrated that actual antiviral effector molecules in the IFN response are a subset of the genes upregulated by IFN, which are called IFN-stimulated genes (ISGs). Thus far, hundreds of genes have been classified as ISGs, and many of them are reported to have inhibitory effects on divergent families of viruses, including flaviviruses [80–83]. Importantly, several ISGs have also been shown to restrict DENV infection, and their suppressive effects are likely to be exerted at the multiple steps of virus replication, including virus entry (IFITMs [84, 85], ADAP2 [86]), viral RNA/protein synthesis (ISG20 [87], viperin [88, 89]), and infectious virion production (tetherin [90], ISG15 [91, 92]). In addition, a comprehensive study using an overexpression of a cDNA library derived from known ISGs demonstrates the involvement of

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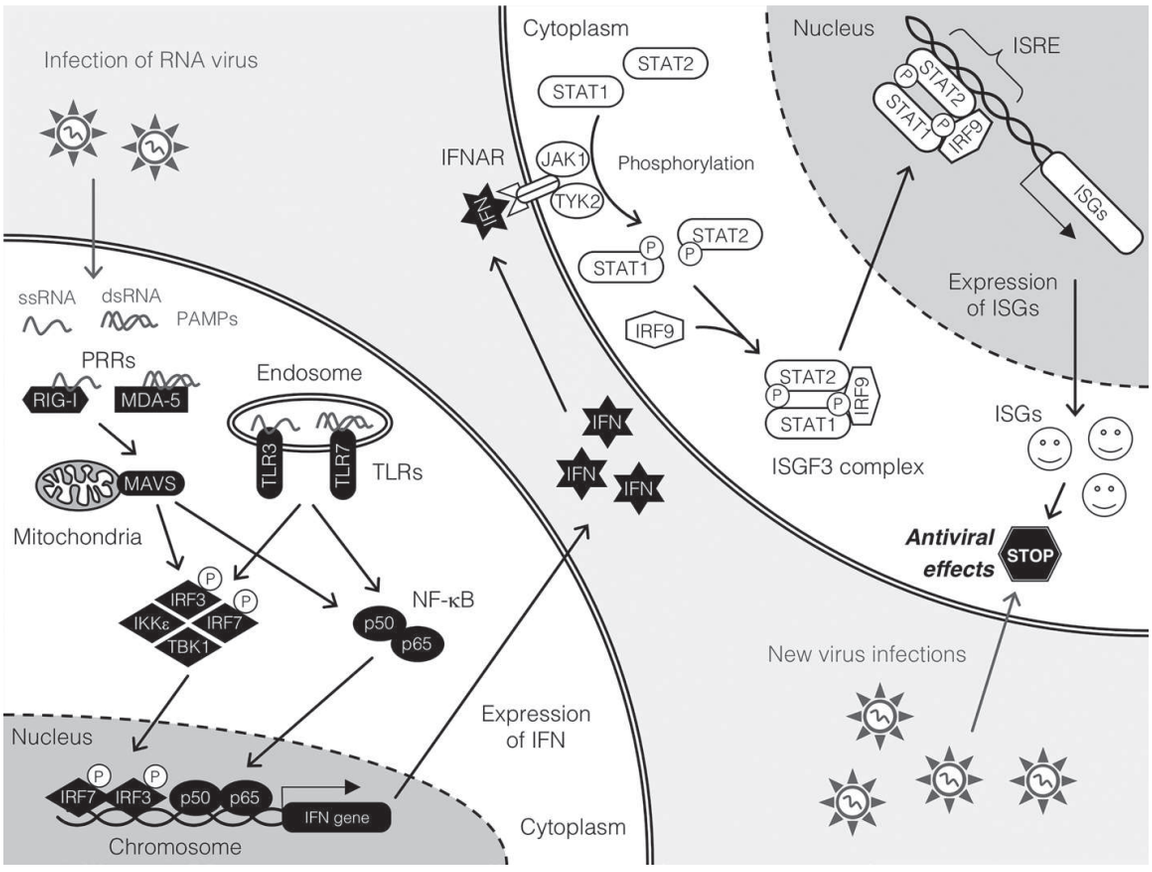

Figure 4. Intracellular recognition of RNA viruses and IFN responses (left). PRRs-sensing virus-specific nucleic acid structures (ssRNA and dsRNA) activate signalling cascades via phosphorylation of transcription factors (IRF3 and IRF7), which leads to the expression of IFN genes together with NF-κB (right). Binding of IFNs produced from virus-infected cells to type I IFN receptor (IFNAR) activates JAK1 and TYK2, which, in turn, phosphorylate STAT1 and STAT2. Phosphorylation of STAT1/2 induces the formation of ISGF3 complex with IRF9, and finally, ISGF3 translocated in nucleus binds to ISREs to induce ISG expressions. ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; MAVS, mitochondrial antiviral-signalling protein; JAK1, Janus kinase 1; and TYK2, tyrosine kinase 2.

many more ISGs in the restriction of DENV [93]. However, the precise mechanisms of these ISG-mediated anti-DENV activities remain unclear, and we will have to wait for future studies for detailed analysis.

6. Identification of RyDEN/C19orf66 as novel anti-DENV ISG

It has been well demonstrated that a gain-of-function (i.e., overexpression) screen of cDNA is a powerful approach to identifying antiviral ISGs [82, 83, 93, 94]. In a recent study, we employed the gain-of-function strategy using a pool of cDNA library derived from type 1 IFN-treated cells to search for ISGs suppressing DENV replication in human cells [95]. In this approach, a human cervical carcinoma cell line, HeLa, was treated with type I IFN (a mixture of human IFN-α and -ω) for 24 hours at a concentration that had been reported to inhibit DENV infection *in vitro* [96], and mRNA from the IFN-treated HeLa cells was converted into a library of cDNA and transferred to a HIV-based lentiviral vector. Then, a human hepatoma cell

line, Huh7.5, was exposed to the HIV vector carrying the IFN cDNA library. Huh7.5 cells are highly susceptible to DENV and, therefore, exhibit massive cell death upon DENV infection. Therefore, we expected that if anti-DENV genes derived from IFN-treated HeLa cells were introduced into DENV-permissive Huh7.5 cells, those cells should be non-permissive and survive DENV-induced cell death. As anticipated, even with the DENV challenge, surviving cell clones were obtained; subsequent sequencing analysis revealed that approximately half of DENV-resistant clones harboured an ORF of a gene on chromosome 19, *C19orf66*, in the integrated HIV vector genome [95]. Since *C19orf66* was a previously uncharacterized gene, we named this *r*epressor of *y*ield of *DEN*V (RyDEN).

The ORF of RyDEN (*C19orf66*) encodes a 291 amino acid protein, and the secondary structure prediction suggested that the RyDEN protein contained a nuclear localization signal (NLS) in the middle region and a nuclear export signal (NES) in the C-terminal region. Additionally, a characteristic glutamic acid (E)-rich domain was found in the C-terminus.

The anti-DENV activity of RyDEN was confirmed by creating stable cell lines that expressed epitope tag-fused human hepatoma cell lines, and all DENV serotypes (i.e., DENV-1–4) were found to be inhibited by RyDEN expressions. In line with the fact that this gene was first identified by the gain-of-function approach using an IFN-derived cDNA library, expressions of RyDEN in various human cell lines were upregulated by IFN treatment to a greater or lesser extent, indicating that RyDEN is a bona fide anti-DENV ISG. More importantly, when the endogenous expression of RyDEN mRNA was knocked down by RNAi, the suppressive activity of type I IFNs against DENV became less effective, suggesting that RyDEN was a major contributor of the IFN-mediated anti-DENV response [95].

One question to ponder: what is the molecular mechanism by which RyDEN suppresses DENV replication? Affinity purification-mass spectrometry analysis with affinity tag-fused RyDEN found that RyDEN interacted with two other cellular proteins, poly(A)-binding protein cytoplasmic 1 (PABPC1) and La motif-related protein 1 (LARP1). PABPC1 is a member of the PABP family; as described above, this protein bridges the 5′ and 3′ ends of mRNA by binding both the eIF4G and the poly(A) tail, which stimulates the initiation of translation [97]. LARP1 is also an RNA-binding protein and one of the LARPS, which shares the signature motif with the La autoantigen, called the La motif (LM). Therefore, Larp and La proteins are categorized as being in the same family [98]. Intriguingly, LARP1 is shown to interact with PABP to stimulate the mRNA translation process [99, 100]. Considering the positive effect of PABP on translation [97], the association of PABPC1 and LARP1 with RyDEN suggests that RyDEN might interfere with the translation of DENV RNA by inhibiting PABPC1 and LARP1 functions. Indeed, this speculation was supported by the following findings: (i) an RNAi-mediated knockdown of PABPC1 and LARP1 significantly reduced the level of DENV replication, (ii) PABPC1 interacted with DENV RNA in infected cells, (iii) the expression of RyDEN suppressed the expression of the reporter protein from a DENV-based sub-genomic RNA replicon that lacked structural (C, prM, E) genes, and (iv) the recruitment of RyDEN to the DENV RNA 3′ UTR was enhanced by the presence of PABPC1 *in vitro* [95]. Therefore, one could envisage that the anti-DENV activity of RyDEN, an antiviral ISG, is exerted during the translation of viral RNA by associating PABPC1/LARP1 with the 3′ UTR and somehow interfering with the function of the translation factors (**Figure 5**). Or another possibility is that

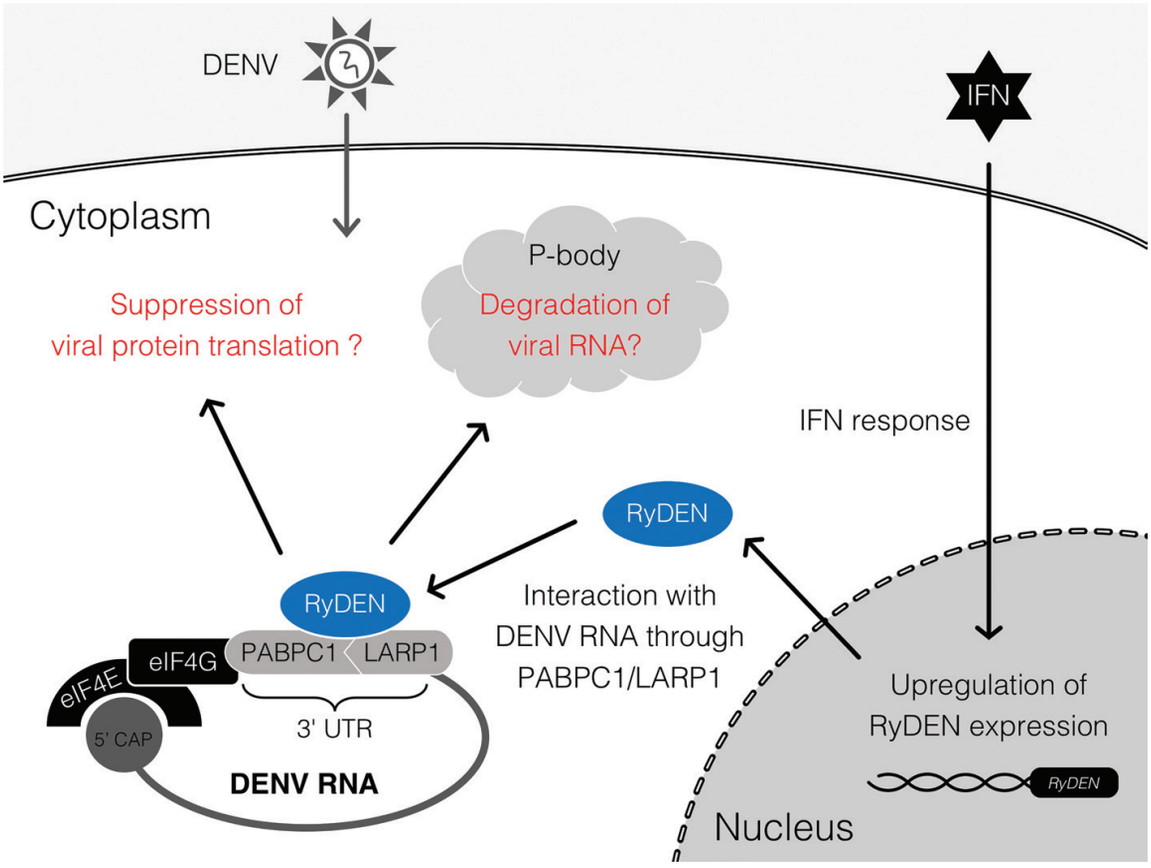

Figure 5. Possible mechanisms by which RyDEN restricts the function of DENV RNA. RyDEN, whose expression is upregulated by IFN, associates with DENV RNA through interaction with PABPC1 and LARP1 that are required for DENV replication. This association may result in (i) translational suppression or (ii) degradation of viral RNA in the cytoplasm.

RyDEN may facilitate the degradation of viral RNA, since PABPC1 and LARP1 have been reported to be involved in eukaryotic mRNA decay as processing body (P-body) and stress granule (SG) components (**Figure 5**) [99, 101]. In accordance with this notion, a recent study revealed that RyDEN (also referred to as FLJ11286 or IRAV) was colocalized with cytoplasmic P-bodies in IFN-treated cells [102]. Furthermore, it was noteworthy that RyDEN expression limited a diverse range of RNA and DNA viruses [83, 95, 102], indicating that RyDEN is a broad-spectrum antiviral ISG.

7. Concluding remarks

This chapter highlighted the molecular interactions between DENV and host factors, particularly focused on the cellular regulation of DENV replication. As is well known, IFN response is one of the host controls of DENV infection and pathogenesis [96, 103, 104]. Although the effector molecules in the IFN response that actually interfere with virus replication remain fully clarified, profound efforts have been made to identify the IFN-inducible cellular factors restricting RNA virus replication, including DENV [80]. It should be noted that antagonistic

effects of DENV infection on IFN signalling and production, which are mediated by several means using viral factors, are observed [105–107]. However, the characterization of anti-DENV ISGs is to illuminate the "heel of Achilles" of DENV, which will provide the underpinnings for the development of antivirals against dengue.

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