Host Factors in the Replication of Positive-Strand RNA Viruses

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Viruses are obligate, intracellular parasites that depend on host cells for successful propagation. Upon infection of host cells, positive-strand RNA viruses exploit and hijack cellular machinery and reprogram these cells into viral "factories" through various protein-protein, protein-RNA, and protein-lipid interactions. The molecular interplay between host factors and invading viruses is a continuous process throughout the entire viral life cycle and determines virus host range and viral pathogenesis, as well as driving viral evolution. Studies of host factors have contributed insights into their normal cellular functions and helped identify attractive targets for antiviral drug development. With the development of high throughput screening, functional genomics, and proteomics technologies, host factors participating in viral life cycles have been identified rapidly in recent years. In this review, we summarize the recent advances in virus-host cell interactions in positive-strand RNA virus infections and focus on host factors that facilitate viral replication. *(Chang Gung Med J 2012;35:111-24)*

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Among the seven different classes of eukaryotic viruses, the positive-strand (+)RNA viruses constitute the largest group and comprise a variety of viruses that infect mammalian, plant, and insect hosts. Among the (+)RNA viruses are many pathogens of importance to human health, e.g., members of the *Flaviviridae* (e.g., hepatitis C virus [HCV], dengue virus [DENV], and Japanese encephalitis virus [JEV], *Coronaviridae* (e.g., severe acute respiratory syndrome coronavirus), and *Picornaviridae* (e.g., poliovirus and coxsackievirus). The (+)RNA viruses also include numerous economically important veterinary and plant viruses. Examples include the pestiviruses of *Flaviviridae* (e.g., bovine viral diarrhea virus and classic swine fever virus) and members of *Arteriviridae* (e.g., equine arteritis virus and porcine reproductive and respiratory syndrome virus) and tobacco mosaic virus. The (+)RNA viruses are characterized by a positive-sense, single-stranded RNA genome, which, upon virus entry and uncoating, functions as mRNAs and thus can be directly translated by host cell

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machinery. The genomic RNA also serves as the template for viral RNA replication. Following translation and processing of the viral polyprotein(s), the viral RNA-dependent RNA polymerse (RdRp), along with other viral nonstructural (NS) proteins, viral RNA, and host factors, form membrane-associated replication complexes (RC) that carry out viral RNA synthesis.[1-3] The resultant progeny (+)RNA strands can either initiate a new translation cycle or be packaged into virions that are subsequently released to infect naïve cells.

As with viruses of other classes, (+)RNA viruses are obligate, intracellular parasites that cannot reproduce outside their host cells. Encoding only a limited number of proteins, (+)RNA viruses have evolved elaborate mechanisms to reprogram host cells for their propagation by exploiting and hijacking host proteins, membranes, lipids, and even in some cases microRNAs and by subverting cellular pathways during infection. Current data suggest that all steps of the (+)RNA virus life cycle require the participation of host factors, including virion entry and disassembly, viral RNA translation, polyprotein processing, viral RNA replication, virion assembly, and release. The molecular interplay between host factors and invading viruses is a continuous process that governs virus host range, tissue specificity, and viral pathogenesis and is a driving force in viral evolution.[4] The study of host factors in the viral life cycle provides insights into their normal cellular functions and helps identify attractive targets for developing new, effective, antiviral drugs.

The recent development of genome-wide high throughput screening technologies and advanced proteomics approaches has greatly facilitated research efforts in delineating host factors participating in the (+)RNA virus life cycle. Genome-scale screens have been performed for a number of (+)RNA virus infections in their respective model host cells, including infections with HCV; West Nile virus (WNV); DENV; plant viruses (Brome mosaic virus, tombusvirus [TBSV]); and Drosophila C virus [DCV], an insect (+)RNA virus.[5-13] These studies have confirmed that (+)RNA viruses rely greatly on intracellular components of infected hosts for viral replication, and have identified novel host factors and pathways important for various steps in the viral life cycle. In this review, we focus on recent advances in host factors facilitating the replication of a few relatively well-studied (+)RNA viruses. Although fundamental differences exist in the mechanism of viral replication, these viruses co-opt some common host factors/pathways, which may represent potential targets for future development of antivirals with broad-spectrum activity.

An overview of viral RNA replication of (+)RNA viruses and interactions with the host

Following translation and processing of viral proteins, (+)RNA viruses adopt a relatively conserved process for completing the RNA replication cycle in host cells. Studies of the mechanisms of (+)RNA virus replication have suggested that this process usually involves the following steps: 1) selecting and recruiting viral (+)RNA templates; 2) targeting viral replication proteins to the replication site, which involves extensive cytoplasmic membrane rearrangements; 3) forming and activating viral RCs in association with virus-induced membrane vesicles/vacuoles; 4) synthesizing progeny viral RNAs, including synthesis of the negative-strand RNA using input (+)RNA as the template and synthesis of the progeny (+)RNA using the intermediate negative-strand RNA as the template; 5) liberating progeny (+)RNAs from vrial RCs for their packaging into virions and subsequent egress; and 6) disassembling the viral RCs (Figure).[4,14-19] Accumulating evidence suggests that the entire RNA replication process of (+)RNA viruses is complex, involving not only interactions of viral RNAs and viral proteins but also their interplay with host factors. Studies performed for infections with HCV, DENV, WNV, and several plant (+)RNA viruses have shown that viral RNA replication requires host membranes, and proteins and lipids involved in various cellular processes, including but not limited to metabolism/modifications of RNAs, lipids and proteins, and intracellular trafficking and targeting of proteins,[6,20-23] some of which will be discussed in detail later in this review. Yet this is not the whole picture, and it has recently been demonstrated that certain cellular microRNAs can be co-opted for (+)RNA virus replication. A prime example is mir-122, a liver-specific microRNA that interacts with the 5'-untranslated region (UTR) of HCV RNA and promotes HCV RNA replication through as-yet-elusive mechanisms.[24] In general, thus far, the identified host factors facilitating the replication of

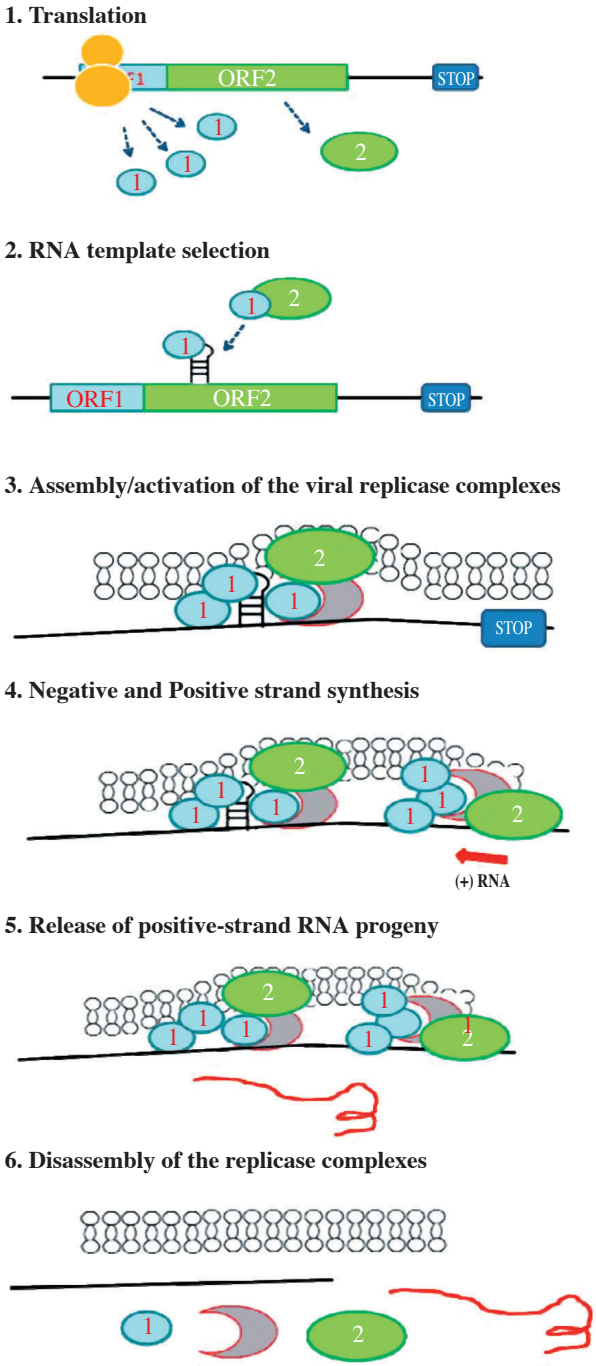

Figure A model of the (+)RNA virus replication pathway. The six separate steps proposed during (+)RNA virus replication are listed, including the viral and putative cellular factors involved in each step. The replicase complexes are shown schematically, but they likely contain more host factor components. Abbreviations used: ORF: open reading frame; (1) & (2): viral proteins 1 & 2.

(+)RNA viruses fall into the following categories, although the exact roles played by most of them are still unclear: (1) those directly involved in completing one or more essential steps of viral replication by directly interacting with and modulating the functions of viral RNAs and/or viral proteins; and (2) those indirectly regulating the viral life cycle by controlling the quantity and availability of proviral cellular factors. It should be noted that genome-scale RNA interference (RNAi) screens have also identified numerous host factors as part of the intrinsic antiviral defense mechanism, the presence of which negatively regulates viral replication.[15,25] This review summarizes current understanding of the relationship between (+) RNA virus replication and the rearrangement of host cytoplasmic membranes, the participation of the cholesterol and fatty acid metabolite pathway, host factors regulating the formation of the viral RNA replication complex, the role of molecular chaperones and host factors affecting the assembly of virus particles. This review also lists several systematic approaches used for identifying host factors regulating the (+) RNA virus life cycle.

The rearrangement of host cytoplasmic membranes for (+)RNA virus replication

Although highly divergent in host range, virion morphology, and genome organization, (+)RNA viruses share a common replication strategy – they dramatically remodel host intracellular membranes into specialized compartments/organelles that foster viral replication.[1,26] The virus-induced membrane organelles help increase the local concentrations of necessary components for viral replication and provide the structural frame for assembly of viral RCs. In addition, they are believed to be exploited by viruses to shield RC components from degradation by cellular nucleases and proteases and to evade recognition by the host innate immune system.

The membrane vesicles/vacuoles induced in (+)RNA virus infected cells usually accumulate in the perinuclear region and appear as spherule-like structures formed by invagination of cellular membranes, although the vesicles induced by certain viruses may appear more heterogeneous in size, such as those seen in cells replicating HCV. The lumen of the vesicles is enclosed but retains access to the cytoplasmic constituents via a pore or neck, providing a

protected microenvironment for viral RNA synthesis.[2,27] However, the membrane origin of the vesicles induced by different viruses may vary, implying different intracellular sites of viral RNA replication. The RCs of members of the picornaviruses, flaviviruses, hepaciviruses, coronaviruses, and arteriviruses associate with membrane vesicles derived from the endoplasmic reticulum (ER), while the membrane sources for togoviruses (e.g, Semliki Forest virus and rubella virus)-induced vacuoles are endosomes and lysosomes. In addition, plant (+)RNA viruses such as TBSV can replicate on either peroxisomes or the ER, depending on membrane availability. Finally, flock house virus, an insect (+)RNA virus classified in the family *Nodoviridae,* assembles its RC on mitochondrial membranes.[1,3]

Genetic analyses have revealed that induction of the specialized membrane organelles can be attributed to a particular viral gene product, usually an NS protein that is a component of the viral RC.[28] In ectopic expression systems, the NS4A of classic flaviviruses, the NS4B of HCV, the 2BC and 3A of picornaviruses, and the nsp3 to nsp4 of arteriviruses and coronaviruses were all reported to induce membrane rearrangements similar to those observed in virus infected cells.[1,29-32] However, how these viral NS proteins induce membrane reorgnization in favor of viral replication is not clear. Two different mechanisms have been proposed for poliovirus induction of the characteristic double membrane vesicles for viral RNA replication. In one theory, poliovirus subverts the autophagy pathway by 2BC- and 3A-mediated recruitment of LC3 to form a complete autophagosome-like vesicle. Subsequently, viral RdRp and other components of viral RC are recruited to these vesicles for viral replication. Importantly, poliovirus may have evolved to block subsequent maturation and degradation of these autolysosomal-like membranes.[33] A second theory involves the subversion of the ADP-ribosylation factor (Arf)- and guanine nucleotide exchange factor-dependent cellular secretory pathway.[34] In this model, poliovirus 3A and 3CD proteins hijack two key components of the cellular secretory pathway, the small GTPase Arf1 and its activator, guanine nucleotide exchange factor GBF1, thereby disrupting the coat protein-dependent cellular vesicle transport normally occurring between the Golgi and ER. A very recent study provided new insight into the detailed mechanism of how 3A proteins of poliovirus and coxsackievirus co-opt this secretory pathway to generate the membranous vesicles for viral replication. It was shown that 3A uses GBF1 and Arf1 to recruit a phospholipid-modifying kinase, PI4K-IIIβ, resulting in the synthesis of phosphatidylinositol 4-monophosphate (PI4P) lipids. PI4P, in turn, facilitates membrane remodeling, as well as the recruitment of viral RdRp and other viral proteins for efficient RNA synthesis.[35] Interestingly, the PI4P-dependent pathway was also found to be essential for forming the membranous HCV RCs,[13,35-37] indicating a conserved mechanism among (+)RNA viruses.

The participation of cholesterol and fatty acid metabolic pathways in (+)RNA virus replication

Cholesterol and fatty acids are essential constituents of cellular membrane lipids that regulate the fluidity, permeability, and integrity of cell membranes. In addition, various lipid groups can be covalently attached to proteins, a process known as protein lipidation. This class of posttranslational modification has been shown to occur with hundreds of proteins and regulates protein trafficking, protein-protein interactions, and protein association with membranes.[38] Because (+)RNA viruses depend on intracellular membranes for their replication, perturbations in membrane lipid composition and/or protein lipidation are likely to impact viral replication. Indeed, a number of studies have highlighted a role for cholesterol and fatty acid biosynthetic pathways in regulating the replication of different (+)RNA viruses.

The cholesterol biosynthetic pathway

Cholesterols can be synthesized from acetyl-CoA via the mevalonate pathway. This pathway also leads to the production of several isoprenoids, including farnesyl pyrophosphate and geranylgeranyl pyrophosphate, both of which are involved in lipidation (prenylation in this case) of proteins.[39] The involvement of the mevalonate pathway for replication of (+)RNA viruses has been relatively well studied in hepatoma cells bearing genotype 1 HCV RNA replicons.[40,41] Ye et al. first reported that HCV RNA replication is sensitive to treatment with lovastatin,[40] a cholesterol-lowering drug that inhibits HMG-CoA

reductase, the rate-limiting enzyme in the mevalonate pathway. The authors found that the inhibition of protein geranylgeranylation, rather than that of farnesylation or cholesterol synthesis, is responsible for disruption of the HCV RC and the suppression of HCV RNA replication by lovastatin. Subsequently, a geranylgeranylated cellular protein, FBL2, was identified to associate with HCV NS5A and to be required for HCV RNA replication, although its exact function in HCV replication remains unclear.[42] Recently, it was demonstrated that replication of WNV and DENV also requires the mevalonate-dependent cholesterol biosynthetic pathway.[43-45] The reliance on the cholesterol synthetic pathway for efficient viral replication is not restricted to mammalian (+)RNA viruses. Genome-wide screens revealed that the sterol biosynthesis genes ERG25 and ERG4 were required to replicate TBSV, a plant (+)RNA virus, in a yeast model host.[46] Downregulation of ERG25 expression or inhibition of its activity severely impaired TBSV RNA replication, as did the sterol biosynthesis inhibitor, lovastatin, confirming that the sterol biosynthesis pathway is required for TBSV replication.

Fatty acid synthase (FASN)

FASN is a key lipogenic enzyme catalyzing the terminal steps in the de novo biogenesis of fatty acids. This 270-kD cytosolic enzyme is responsible for synthesizing the 16-carbon fatty acid, palmitate, from malonyl-CoA, using acetyl-CoA as a primer.[47] Prior to the early 1990s, the involvement of lipid biosynthesis in the replication of (+)RNA viruses was appreciated in experiments using a fatty acid synthesis inhibitor, cerulenin. It was shown that, when lipid synthesis was blocked by cerulenin in HeLa cells, the replication of Semliki forest virus, an alphavirus, was severely compromised.[48] Cerulenin was also reported to suppress poliovirus RNA synthesis in vitro in HeLa cell extracts,[49,50] likely through inhibiting uridylylation of the picornaviral VPg protein primer.[49] Confirming the proviral role of fatty acid synthesis in picornavirus replication, a recent study demonstrated that FASN was strongly induced in Coxsackievirus B3 (CVB3)-infected HeLa and HepG2 cells compared with uninfected cells revealed by 2-dimensional electrophoresis and subsequent mass spectrometry. When the infected cells were treated with different FASN inhibitors, CVB3 replication was severely compromised.[51] Direct evidence for the essential role of FASN in (+)RNA virus replication came from studies with DCV, HCV, and DENV. DCV is a picornavirus-like (+)RNA virus belonging to the family *Dicistroviridae*. A recent genome-wide RNAi screening showed that CG3523, the gene encoding FASN in *Drosophila,* is required for DCV replication in this host species. Knocking down CG3523 abrogated DCV replication, as well as the formation of DCV-induced of membrane vesicles.[52] In HCV-infected hepatoma Huh7 cells, FASN expression was upregulated.[53] FASN is required for HCV propagation, as RNAi knockdown of FASN or inhibition of FASN activity by cerulenin or C75 suppressed HCV replication in genotype 1 HCV RNA replicon-bearing cells, as well as in cells infected with the genotype 2a infectious virus.[53,54] Interestingly, the study by Yang et al. demonstrated that C75 not only inhibited HCV RNA synthesis but also downregulated the expression of claudin-1,[53] a tight junction protein that serves as a coreceptor for HCV entry. By RNAi screening, Heaton et al.[55] identified FASN as an essential cellular factor for DENV replication. The authors confirmed their observation by using various FASN inhibitors and went on to extend this finding to two other flaviviruses, yellow fever virus (YFV) and WNV. However, the mechanism was fundamentally different from that proposed for HCV. Instead of increasing FASN expression, DENV NS3 interacts with and recruits FASN to viral RCs and stimulates FASN activity, thereby promoting viral replication.[55]

Host factors regulating the formation of the viral RNA RC

As discussed above, replication of (+)RNA viruses takes place on reorganized intracellular membraneous compartments. These specialized membrane organelles house the membrane-bound viral RCs that perform many functions during viral RNA replication, including recognition of minus- and plus-strand initiation promoters located at the 3'terminus of a plus- or minus-RNA template, and de novo (primer-independent) or primer-dependent initiation, as well as synthesis of complementary RNA strands, strand separation, and repair of viral RNAs with damaged termini.[56] Although the manner in which the viral RC is able to carry out so many activities is poorly understood, the multifunctionality

is mostly likely attributed to the elaborate composition of the viral RC, which consists of both viral- and host-derived components. The current model suggests that formation of an active (+)RNA virus RC requires at least two components, (1) highly organized protein-protein and protein-RNA complexes and (2) cellular membranes.[17,57] Host factors hijacked/co-opted by viruses may contribute to the formation of viral RC by facilitating membrane rearrangements and recruiting essential viral and cellular proteins to the membranes.[26] Moreover, host factors in the viral RC may regulate the activities of viral enzymes. It is known that some viral RdRps, such as the HCV NS5B and the poliovirus $3D^{pol}$, are nonfunctional prior to their incorporation into the RC. Here, we discuss the contributions of several cellular factors in the formation of the HCV RC.

The assembly of HCV RC was reported to take place on detergent-insoluble lipid rafts, which consist of cholesterol- and sphingolipid-rich microdomains within the subcellular membranes. The human vesicle-associated membrane protein-associated protein A (hVAP-A, also known as hVAP-33), an ER-Golgi-localized protein involved in intracellular membrane trafficking, was shown to interact with HCV NS5A and NS5B and to play a critical role in the assembly of HCV RC on lipid rafts.[58,59] hVAP-A is partially associated with lipid raft membranes and facilitates the recruitment of NS5B to join NS4A-NS4B-NS5A and NS3 on the lipid raft. The overexpression of dominant-negative hVAP-A mutants or RNAi-mediated depletion of hVAP-A resulted in relocation of NS5B from detergent-resistant to detergent-sensitive fractions. Interestingly, Evans et al. found that there is an inverse correlation between HCV NS5A phosphorylation and hVAP-A interaction.[60] Hypophosphorylated NS5A can interact with hVAP-A leading to efficient replication, whereas NS5A hyperphosphorylation disrupts its association with hVAP-A and negatively regulates viral RNA replication. Thus, host factors regulating the phosphorylation of NS5A may impact the HCV life cycle by modulating the ability of NS5A to participate in critical protein-protein interactions that regulate the switch from HCV RC assembly and viral RNA replication to later viral life cycle events, such as RC disassembly and virion packaging.

Estrogen receptor (ESR) is a member of the steroid hormone receptor family of the nuclear receptor superfamily. ESR mainly resides in the cytoplasm under normal conditions. Upon activation by estrogen, ESR translocates into the nucleus, where it serves as a transcription factor to activate downstream gene expression. Two forms of ESR exist, ESRα and ESRβ, each encoded by a separate gene. Recently, a role for ESRα in regulating HCV RC formation was reported.[61] It was found that a fraction of ESRα localizes to ER membranes, where it interacts with the HCV RdRp, NS5B. This association promotes the incorporation of NS5B into the HCV RC, thereby facilitating HCV replication. Knocking down ESRα expression inhibited HCV RNA replication. A similar effect was also observed in cells treated with tamoxifen or other anti-estrogen drugs.

HCV may also exploit/hijack the endocytic trafficking machinery for assembly of its RC on the membranous web. This notion was first suggested by the observation that Rab5, an early endosome-localized small GTPase involved in membrane fusion, colocalizes and associates with HCV NS4B, the viral protein responsible for inducing the membraneous web. Disruption of Rab5 function by overexpression of a dominant negative Rab5 or RNAi knockdown of Rab5 expression substantially compromised HCV RNA replication, and remarkably, disrupted the formation of the membraneous web.[62] In another study, Berger and colleagues conducted a focused RNAi screen targeting 140 host membrane trafficking genes and identified additional endocytic genes required for HCV RNA replication and infectious virus production.[36] These include the early endosomal protein EEA1 and late endosome protein Rab7. The involvement of host factors in the endocytic pathway in HCV replication is reminiscent of alphaviruses such as the Semliki Forest virus, the replication of which was reported to operate on endosomal membranes.[63]

Recently, the PI4K-IIIα lipid kinase has emerged as a crucial cellular factor for HCV RC formation. Encoded by the PIK4CA gene, PI4K-IIIα resides primarily in the ER, and its main cellular function is to generate PI4P lipids. PI4K-IIIα was first identified in 2004 as an interaction partner of HCV NS5A in yeast two-hybrid assays, with no attributed function.[64] In 2009, multiple research groups reported the identification of PI4K-IIIα as an essential cellular factor for HCV replication by RNAi screening.[5,13,36,65,66] Remarkably, silencing the

expression of PI4K-IIIα resulted in aberrant localization of NS5A and diminished the formation of the membranous web where HCV RC assembles. The current model is that NS5A interacts with PI4K-IIIα and stimulates its kinase activity to synthesize PI4P lipids, which promotes the formation of the membranous HCV RC. Interestingly, the PI4P-dependent formation of membrane organelles for viral RNA replication was also observed for two picornaviruses, poliovirus and CVB3, although these viruses seem to prefer exploiting PI4K-IIIβ over PI4K-IIIα.[35]

Roles of molecular chaperones in viral RNA replication

Molecular chaperones represent a large number of heat shock proteins and protein-remodeling factors, which help proteins obtain their active conformations and mediate refolding and/or degradation of trapped, misfolded proteins.[67-69] The best known function of molecular chaperones is to mediate protein quality control and maintain protein homeostasis in cells. In virally infected cells, however, molecular chaperones can be subverted by viruses for folding viral proteins during and after translation and for controlling the stability of viral proteins. In this review, we discuss only the specialized exploitation of the heat shock proteins (hsp) and cyclophilins (CyP) for viral RNA replication.

The heat shock protein (hsp)70 and its co-chaperone, hsp40, are required for HCV replication.[70] These two proteins were co-purified with HCV NS5A in cell extracts and identified by mass spectrometry. RNAi knockdown of hsp70 and hsp40 impaired HCV replication in an HCV cell culture system, as did quercetin, a specific inhibitor of hsp synthesis. It was proposed that quercetin may inhibit HCV replication by reducing the expression of hsp70 and hsp40 and their potential involvement in HCV internal ribosome entry site (IRES)-mediated translation.[70] Another hsp70 family member, hsp72, was also reported to facilitate HCV RNA replication. Chen et al. found that hsp72 interacted with three HCV proteins, NS5A, NS3, and NS5B (RdRp), all of which are components of the HCV RC.[71] Downregulation of hsp72 expression led to decreased HCV RNA replication, while overexpression of Hsp72 had the opposite effect. Hsp72 was found to increase the levels of HCV RC by augmenting the stability of HCV replication proteins and/or enhancing their translation via the HCV IRES.

Hsp90, one of the abundant chaperones in cells, is also required for HCV replication. Hsp90 interacts with NS3 and enhances its stability. It also forms a complex with HCV NS5A and the host FKBP8 immunophilin.[72,73] The human butyrate-induced transcript 1 (hB-ind1), an hsp90 co-chaperone-like analogue protein, colocalizes with HCV dsRNA, NS5A, and FKBP8 on the membraneous web in HCV replicon cells, whereby it interacts with NS5A and recruits hsp90 for HCV replication.[74] Based on the known functions of hsp90 and its co-chaperones, it was proposed that hsp90 and hB-ind1 are recruited to the HCV RC to promote the correct folding of the HCV replication proteins, to prevent the induction of unfolded protein responses, and to facilitate the intracellular movement of HCV-hijacked cellular membranes. Hsp90 and hB-ind1 might also affect the phosphorylation status of NS5A, which is known to regulate the role of NS5A in HCV replication.[74]

The cyclophilins (CyPs) are a family of peptidyl prolyl isomerases (PPIase) that also possess protein chaperone-like activities. CyPs catalyze the isomerization of peptide bonds from the trans form to the cis form at proline residues and facilitate protein folding.[75] A role for CyPs in the replication of HCV and the classic flaviviruses was revealed recently. The first clue came from studies showing that the replication of HCV RNA replicons was sensitive to treatment with cyclosporine A (CsA), an immunosuppressive drug that inhibits CyP function.[61,76] However, conflicting results have been reported as to which CyP subtype is essential for HCV replication. CyPA, CyPB, and CyPC were all suggested to be indispensible, but there is a growing consensus more recently that CyPA is the main isoform crucial for HCV replication[77-79] and the principal mediator of CsA resistance.[77,78] Although the PPIase motif of CyPA has been shown to be important,[78-80] the mechanism by which CyPA regulates HCV replication remains unclear. NS5B, NS5A, and NS2 have all been proposed to be potential targets of CyPA,[81-84] but there is compelling evidence that the association of CyPA with domain II of NS5A is important for HCV replication.[85,86] Interestingly, CyPA also forms a complex with NS5 of the classic flaviviruses. Disruption of this association by CsA strongly inhibits the replication of DENV, YFV, and WNV, suggesting that host CyPA is likely a component of

flaviviral RC and could be targeted for potential antiviral development.[87]

Because of their error prone replication, RNA viruses evolve rapidly. Many antiviral drugs and vaccines are no longer effective against the newly emerging resistant viruses. However, targeting host factors required for RNA virus propagation could be an attractive approach for developing antivirals with broad and durable activity. For example, pharmacological inhibition of hsp90 impaired the replication of HCV,[88] poliovirus, rhinovirus, and coxsackievirus in cell culture and in infected animals.[89] Importantly, anti-Hsp90 treatment did not lead to the emergence of drug-resistant escape mutant viruses.[90] The use of quercetin as an anti-hsp70 compound also inhibited the multiplication of HCV and several plant viruses, TBSV, Tobacco mosaic virus, Turnip crinkle virus, and Potato virus A.[70,91,92] Cyclophilin-targeting drugs are known to inhibit the replication of HCV, DENV, YFV, and WNV, and at least for HCV, establishment of viral resistance in vitro was not easy.[86] Taken together, targeting host chaperones might represent a general, broad antiviral strategy.

Host factors affecting the assembly of (+)RNA virus particles

Although a large body of data has accumulated on the mechanisms of viral RNA replication in recent years, relatively little is known about the assembly and release of infectious virions in most (+)RNA virus infections. Nonetheless, there is growing evidence that host factors are involved in virus particle assembly and that some cellular proteins themselves are packaged in the virions and regulate infectivity.

HCV is a paradigm that exploits the lipid metabolism of its host cell, the hepatocyte, for the assembly step in its life cycle. The HCV core protein accumulates around the lipid droplets (LD), which are intracellular organelles for storing neutral lipids.[93,94] The association between HCV core and LDs plays a key role in recruiting NS5A and other viral proteins and for virion assembly.[95] The assembly and release of HCV particles were also shown to be tightly linked to very low density lipoprotein (VLDL) biogenesis,[96,97] which is a crucial pathway present in hepatocytes for maintaining mammalian lipid homeostasis. One of the main protein components of VLDL, apolipoprotein E (ApoE), was shown to interact with HCV NS5A and to be essential for HCV particle formation and release, without affecting viral RNA replication.[97,98] ApoE is also incorporated in HCV virions and is essential for HCV infectivity.[96,99] The role played by another VLDV apolipoprotien, ApoB, however, is controversial and remains to be further investigated.

Several molecular chaperones were recently implicated in viral particle formation during flavivirus infections. GRP78 (also known as HSPA5 or BiP) is a member of the hsp70 family that is localized in the ER lumen. GRP78 is involved in the folding and assembly of proteins in the ER and regulates protein transport through the cell. GRP78 was recently shown to interact with the envelope proteins of DENV and JEV and to be required for virion production and release of these two flaviviruses.[100,101] The effect is specific for the virion assembly/maturation step as depletion of GRP78 had little impact on viral RNA replication. Another constitutively expressed hsp family member, Hsc70, was found to associate with HCV virions produced in cell culture and those isolated from a hepatitis C patient.[102] Hsc70 is not required for HCV replication. Rather, hsc70 colocalizes with HCV proteins around LDs and contributes to controlling LD size and infectious virus production. Anti-hsc70 antibodies decreased HCV virion infectivity, indicating that a fraction of hsc70 is displayed on the surface of HCV virions.

Approaches for identifying host factors that regulate (+)RNA virus life cycle

Because (+)RNA viruses can potentially exploit a large fraction of the 20,000 – 30,000 host proteins for their propagation,[103] it is difficult to identify the cellular proteins that are actually hijacked by a given (+)RNA virus. Many different approaches have been developed over the years to identify host factors regulating viral life cycles. Because of space limitations, we discuss below only a few selected approaches that have been most effective.

Systematic RNAi screen

Genome-scale or genome-wide RNAi screening is of little doubt the most powerful approach in identifying host factors involved in the (+)RNA viral life cycle. Cherry et al. conducted a dsRNA-based, genome-scale RNAi screen in Drosophila to search for host factors affecting the replication of DCV, an

insect +RNA virus. The authors identified 112 genes among the 21,000 genes (91% of the Drosophila genes) targeted. The most interesting finding of this study was that more than half of the identified genes were ribosomal genes, suggesting that replication of DCV relies greatly on the host translation machinery.[11] Subsequent analysis of the identified nonribosomal genes revealed that the coat protein complex I coatamer and fatty acid biosynthesis pathways are also required for DCV replication.[52] The first large-scale RNAi screening for host factors essential for mammalian virus replication was performed with HCV in cells bearing genotype I HCV replicons.[104] Among the 4,000 human genes screened, Ng et al. identified 9 genes whose depletion reduced HCV RNA replication by 60% or more. Interestingly, multiple genes of the tumor necrosis factor/lymphotoxin signaling pathway were found to be required for HCV RNA replication, suggesting a proviral role of NF-κB in the HCV life cycle. Recently, several genome-wide RNAi screens were conducted in HCV replicon-bearing cells or in cells infected with cell culture-derived HCV.[5,13] These studies led to identifying numerous cellular proteins/pathways indispensable for the HCV life cycle, among which was the PI4K-IIIα kinase that is pivotal for HCV RC formation. Of note, 14 human genes involved in the HCV life cycle overlap with those previously identified in an RNAi screen for human proteins required for WNV infection,[6] which may represent important shared host pathways used by the family Flaviviridae.

Forward chemical genetics

This is a useful method for investigating various biological pathways by using exogenous chemical ligands. It involves screening synthetic molecules in cells or organisms for phenotypic changes, the selection of a molecule that produces a phenotype of interest, and the eventual identification of the protein target(s) of the small molecule.[105] This approach has been proven useful for analyzing not only cellular physiological processes but also the molecular mechanisms of viral life cycles.[106] Watashi et al. adopted a cell-based assay to screen compounds that inhibited HCV RNA replication and identified CsA and tamoxifen as potent anti-HCV molecules. Subsequent efforts led to the discovery of the CyPs and ESRα as essential host factors for HCV replication.[107,108]

Proteomics

Recent advances in mass spectrometry methods coupled with the development of proteomic approaches have greatly facilitated the analyses of components of virions and viral RC, protein interactions in infected cells, and virus-induced changes in the cellular proteome, all leading to a more comprehensive understanding of viral infection and viral pathogenesis.[109] To identify host proteins associated with the Sindbis virus (SIN) nsP3 protein, a component of the alphavirus RC, a mutant virus was used to express the viral nsP3 protein tagged with green fluorescent protein (GFP), followed by immunoaffinity purification with anti-GFP antibody and mass-spectrometry identification of the isolated proteins.[110,111] These experiments led to the identification of numerous cellular proteins, many of which were specific interaction partners of SIN nsP3. Recently, two-dimensional gel electrophoresis and mass spectrometry were used to identify host proteins whose expression was altered in DENV-infected K562 and HepG2 cells, respectively.[112,113] In the study conducted by Wati et al., an ER chaperone protein, GRP78, was found to be upregulated by DENV infection and to be necessary for viral antigen accumulation and production of virions,[112] demonstrating the value of this approach in searching for host factors in the flaviviral life cycle.

Yeast two-hybrid (YTH) screens

A large number of studies have used YTH screens. Here, we give only a few examples of YTH screens used to study HCV-host cell interactions. NS5A is an essential component of the HCV RC and regulates a variety of host intracellular signaling pathways through protein-protein interactions. Many HCV NS5A interaction partners were first identified through YTH screening. To investigate the role of NS5A in HCV replication, Tu et al. searched for cellular proteins interacting with NS5A by YTH screening of a human hepatocyte cDNA library. This work identified hVAP-A, which was subsequently demonstrated to be an essential cellular factor for HCV RC formation.[59] Also using YTH assay, Evans confirmed the HCV NS5A-hVAP-A interaction and identified ApoE as an interaction partner with NS5A.[60] The ApoE-NS5A interaction was corrobo-

rated subsequently by multiple research groups via different approaches and was demonstrated to be crucial for HCV virion assembly. The first observation of the interaction between the lipid kinase PI4K-IIIα and HCV NS5A was also made through YTH screening.[64]

Copurification of RNA-protein complexes

As one of the most popular means of identifying RNA-binding proteins, this approach is based on purifying a viral RNA probe incubated with cellular extracts (with or without ultraviolet crosslinking), followed by mass spectrometry-based identification of associated host proteins. A favorite region in viral RNA is the 5'- or 3'-UTR, because these portions of the genome contain the essential cis-replication signals required for viral RNA replication and are known to interact with host proteins. Many RNA-binding host factors involved in coronavirus replication were identified using this approach;[114] examples include the heterogeneous nuclear ribonucleoprotein (hnRNP) A1, polypyrimidine-tract-binding protein, and poly (A)-binding protein. As another example, this approach was useful in revealing that hnRNP-C interacts with the minus-stranded RNA of poliovirus and promotes viral RNA synthesis.[115]

Conclusion and future perspectives

Since the introduction of genome-wide screening approaches and the development of advanced proteomics and functional genomics technologies, large numbers of host factors important for (+)RNA virus life cycles have been rapidly identified. It is expected that knowledge of the number of host factors/pathways involved in the replication of different (+)RNA viruses will continue to grow in the years to come. The emerging information on host-virus interactions contributes to a better understanding of the pathogenesis of various infectious diseases caused by (+)RNA viruses, as well as of the biological functions of cellular proteins/pathways. The information will also help identify attractive targets for future development of antiviral drugs, given that targeting host factors has the advantage of a higher genetic barrier to the emergence of viral escape mutants. While much exciting progress has been made, in-depth analysis of the exact roles for most of the identified host factors in (+)RNA virus life cycles is still scanty. Hopefully, unraveling crucial details in this aspect will increase with the combined use of genetics, biochemistry, and cell biology, which will undoubtedly facilitate the development of new effective measures for controlling diseases associated with (+)RNA virus infections.

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