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EDITORIAL

Roles of the two distinct proteasome pathways in hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) infection often causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The development of a HCV cell culture system enabled us to investigate its whole HCV life cycle and develop a better understanding of the pathogenesis of this virus. Post-translational modification plays a crucial role in HCV replication and in the maturation of viral particles. There is growing evidence also suggesting that the ubiquitin-proteasome pathway and the ubiquitin-independent proteasome pathway are involved in the stability control of HCV proteins. Many viruses are known to manipulate the proteasome pathways to modulate the cell cycle, inhibit apoptosis, evade the immune system, and activate cell signaling, thereby contributing to persistent infection and viral carcinogenesis. The identification of functional interactions between HCV and the proteasome pathways will therefore shed new light on the life cycle and pathogenesis of HCV. This review summarizes the current knowledge on the involvement of the ubiquitin-dependent and -independent proteasome pathways in HCV infection and discusses the roles of these two distinct mechanisms in HCV pathogenesis.

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Key words: Hepatitis C virus; Ubiquitin; Proteasome; Degradation; Hepatitis

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INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus from the family *Flaviviridae* and is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma[1-5]. More than 170 million people worldwide are chronically infected with HCV[6]. The 9.6-kb HCV genome encodes a unique open reading frame encoding a large precursor polyprotein, which is cleaved co-translationally into at least 10 proteins by two viral proteases and two cellular signalases[4,5,7-10].

The previous establishment of a HCV cell culture system has facilitated studies of the whole viral life cycle[11-13]. The HCV life cycle is tightly regulated by both viral and cellular proteins[5] and evidence is accumulating to show that the stability of HCV proteins is regulated through both the ubiquitin-dependent and ubiquitin-independent proteasome pathways[14-18]. Moreover, HCV infection has been shown to trigger the degradation of host factors[19]. It is well known that many viruses manipulate the ubiquitin-proteasome pathway to promote their propagation by redirecting the cellular ubiquitin machinery to enable replication, egress and evasion of the host immune system[20]. Although the majority of the protein turnover mediated by the proteasome occurs through the canonical ubiquitin-dependent 26S proteasome pathway, a number of viral proteins and host proteins are degraded

through the 20S proteasome without prior polyubiquitylation[21,22]. The functional differences between these two proteasome pathways are poorly understood, although a number of proto-oncogenes and tumor suppressors are degraded through both mechanisms, indicative of a system that tightly regulates the turnover of key cellular proteins[23-28].

Ubiquitin is a 76 amino acid polypeptide that is highly conserved among eukaryotic organisms. The ubiquitin/26S proteasome pathway is composed of an enzymatic cascade that ubiquitylates proteins to target them for proteasomal degradation. The E1 ubiquitin-activating enzyme binds ubiquitin through a thioester linkage in an ATP-dependent manner[29,30]. The activated ubiquitin is then transferred to the E2 ubiquitin-conjugating enzyme which works in conjunction with the E3 ubiquitin ligase, which is responsible for conferring substrate specificity[31]. E3 mediates the transfer of ubiquitin to the target protein which is then rapidly degraded by the 26S proteasome[32,33]. A number of studies have revealed the existence of a proteasome-dependent but ubiquitin-independent pathway for protein degradation. Several key molecules, such as p53, p73, c-fos, p21, SRC-3, and the hepatitis B virus X protein are targeted by two distinct degradation pathways that function in a ubiquitin-dependent and ubiquitin-independent manner, respectively[21-28,34,35]. Although the pathophysiological significance of the proteasomal degradation of the HCV proteins and HCV-induced proteasomal degradation of host proteins remains to be elucidated, evidence is accumulating that the proteasome plays an essential role in propagation of HCV[14,15]. The roles of the proteasome pathways in HCV life cycle as well as in viral pathogenesis are further discussed below.

UBIQUITIN-DEPENDENT DEGRADATION OF HCV PROTEINS BY THE PROTEASOME

HCV core protein

The HCV core protein is a major component of the viral nucleocapsid and is a multifunctional factor involved in both the pathogenesis and hepatocarcinogenesis of HCV and is degraded through the ubiquitin-proteasome pathway[5,16,36]. The cellular ubiquitin ligase E6AP was identified as a HCV core-binding protein in our laboratory and shown to mediate the polyubiquitylation of the core protein and thereby target it for proteasomal degradation[14]. E6AP was first identified as the cellular factor that mediates the ubiquitin-dependent degradation of the tumor suppressor p53 in conjunction with the E6 protein of the cancer-associated human papillomavirus types 16 and 18[37,38]. The region between amino acids 58 and 71 of the HCV core protein is responsible for the interaction with E6AP. These 14 amino acids are highly conserved, with the first nine amino acids (PRGRRQPIP) present in the core proteins of all HCV genotypes. This suggests that the E6AP-dependent degradation of HCV core protein is also conserved. Indeed, a knockdown of endogenous E6AP by siRNA increases the production of infectious HCV particles, further suggesting that E6AP negatively regulates HCV propagation[14].

E2 protein

The HCV envelope proteins comprise two glycoproteins, E1 and E2. HCV infection requires the interaction between these proteins and the host cell membrane. HCV attachment and entry into host cells is a multistep process, involving several cell surface molecules, including CD81[39], the LDL receptor[40], scavenger receptor BI[41], claudin-1[42-44], and occludin[43,45]. Several E2 domains also play crucial roles in virus entry[46]. In addition, HCV E2 has been implicated in conferring resistance to interferon (IFN)-α. E2 contains a region homologous to the double stranded RNA-activated protein kinase (PKR) and its substrate, subunit α of the translation initiation factor eIF2α[27]. The unglycosylated form of the E2 protein (E2-p38) is retained in the cytosol and is degraded through the ubiquitin-proteasome pathway[47]. E2-p38, but not the glycosylated form of E2, interacts with PKR and is stabilized by treatment with IFN-α, suggesting that it contributes to the resistance of HCV to IFN-α. The ubiquitin ligase that targets E2-p38 remains to be identified.

NS5A protein

NS5A protein is a major component of the HCV replication complexes and can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms. NS5A harbors an amphipathic α-helix at its amino terminus that promotes membrane association. In addition to this helix region, NS5A contains three domains (Ⅰ-Ⅲ). The N-terminal domain (domain Ⅰ) coordinates a single zinc atom per protein molecule[48]. Zinc mesoporphyrin (ZnMP) is a non-heme metalloporphyrin and a synthetic heme analog of the central zinc in the mesoporphyrin macrocycle. ZnMP enhances the polyubiquitylation and proteasomal degradation of NS5A and suppresses HCV RNA replication[49]. The physiological role of the ubiquitin-dependent proteasomal degradation of NS5A protein is still unclear and the ubiquitin ligase that targets NS5A also remains to be identified.

NS5B protein

The HCV NS5B protein functions as an RNA-dependent RNA polymerase. NS5B binds to a ubiquitin-like protein, hPLIC1[50], which contains 589 amino acids and belongs to a family of type 2 ubiquitin-like (ubl) proteins. hPLIC1 harbors a non-cleavable ubiquitin-like (ubl) domain in its amino terminus and a ubiquitin-associated (uba) domain in its carboxyl terminus[51] and physically associates with the proteasome and at least two ubiquitin ligases (E6AP and βTRCP). NS5B binds to the uba domain of hPLIC1, an interaction which enhances the polyubiquitylation and proteasomal degradation of NS5B, suggesting that

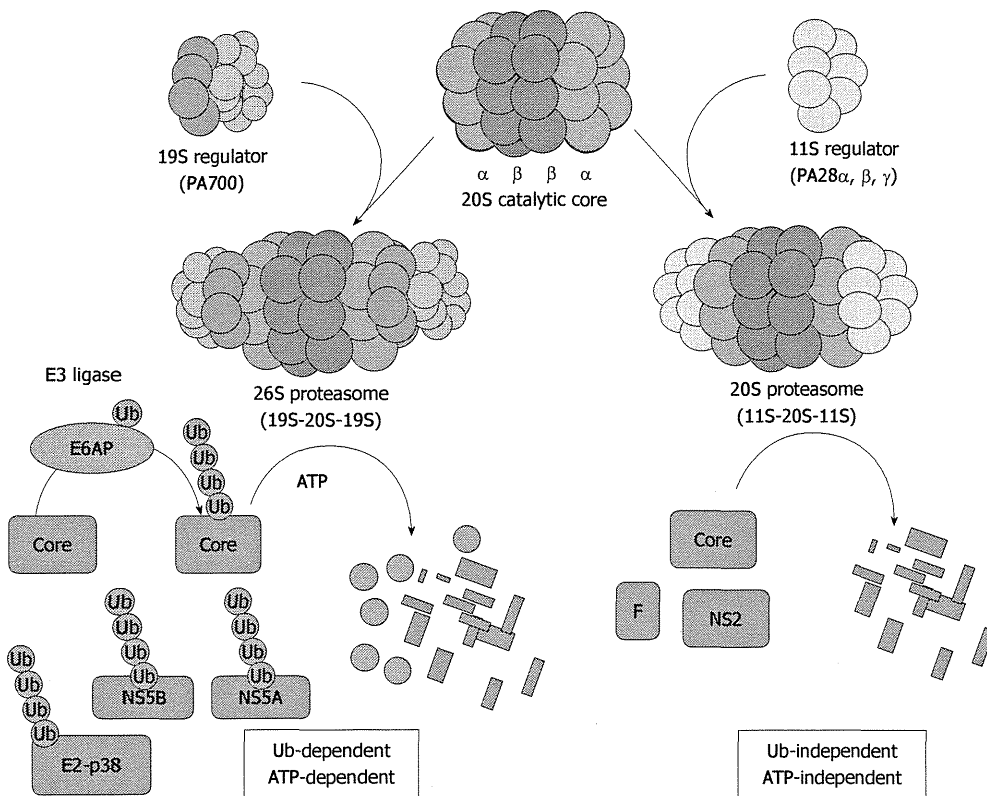

Figure 1 Two distinct proteasome pathways target hepatitis C virus proteins for degradation. The 20S catalytic core is composed of α and β subunits that form a barrel-like structure. The 19S regulator (PA700) can associate with either or both ends of the 20S catalytic core. The combination of one 20S catalytic core and one or two 19S regulator generates the 26S proteasome that is responsible for ubiquitin-dependent ATP-dependent degradation of specific target substrates. E6AP mediates the polyubiquitylation of the hepatitis C virus (HCV) core protein and thereby targets it for ubiquitin-dependent degradation. E2-p38, NS5A, and NS5B are degraded through this ubiquitin-dependent and ATP-dependent proteasome pathway. The proteasome activator, PA28γ, forms a homoheptamer and is implicated in the ubiquitin-independent turnover of the HCV core protein. The F and NS2 proteins are also degraded through the ubiquitin-independent pathway.

hPLIC1 regulates HCV RNA replication by affecting NS5B turnover[50]. The responsible E3 ligase for NS5B ubiquitylation again remains to be identified.

UBIQUITIN-INDEPENDENT DEGRADATION OF HCV PROTEINS *VIA* THE PROTEASOME

Core protein

The HCV core protein specifically interacts with a proteasome activator PA28γ/REGγ in the nucleus and is degraded through a PA28γ-dependent proteasome pathway[18]. *In vivo* experiments in a mouse model have suggested that PA28γ plays a critical role in HCV-associated insulin-resistance, steatogenesis, and hepatocarcinogenesis[52,53]. The proteasomal turnover of the HCV core protein is regulated by two distinct mechanisms, the E6AP-mediated ubiquitin-dependent pathway and the PA28γ-mediated ubiquitin-independent pathway[14,17] (Figure 1). E6AP enhances the ubiquitylation and degradation of the wild-type HCV core protein, but not a lysine-less mutant counterpart, whereas PA28γ enhances the degradation of both the wild type and lysine-less HCV core protein. A knockdown of either E6AP or PA28γ results in the stabilization of the wild-type core protein. However, the knockdown of PA28γ but not E6AP stabilizes the lysine-less mutant core protein, strongly suggesting that PA28γ enhances the ubiquitin-independent pathway. Knockdown of PA28γ in cells at pre-infection or post-infection with the HCV JFH1 strain impaired viral particle production but exhibited no effect on viral RNA replication[15]. The knockdown of PA28γ enhances the polyubiquitylation of the core protein and impairs HCV production, whereas a knockdown of E6AP reduces polyubiquitylation of core protein and enhances virus production. These findings suggest that HCV production is positively regulated by PA28γ and negatively regulated by E6AP through the degradation of the core protein.

F protein

The HCV F protein is encoded by the +1/-2 reading frame encompassed in the 5' end of the polyprotein coding sequence[54]. The F protein is also known as ARFP (alternative reading frame protein) or as core+1 (which indicates the position of the new ORF)[55]. Translation of the F protein is mediated by a ribosomal frameshift at core protein codons 9-11 (HCV-1a strain). The biological role of the F protein remains to be clarified. The F protein is also highly unstable. It has been suggested that the F protein may bind to the α3 subunit of the 20S proteasome[56]. The α3 subunit facilitates the degrada-

tion of the F protein in a dose-dependent manner and a knockdown of the α3 subunit results in the stabilization of the F protein, even in the presence of a replicating HCV genome. The α3-binding domain within the F protein was mapped to the region between amino acids 40 and 60. There are currently three lines of evidence suggesting that the degradation of the F protein is ubiquitin-independent. First, an F protein mutant lacking lysine residues, which therefore cannot be ubiquitylated, is no more stable than the wild-type F protein. Second, F protein expressed in ts85 cells, which harbor a temperature-sensitive E1 ubiquitin-activating enzyme, is not stabilized when the cells are incubated at the non-permissive temperature. Third, the F protein can be degraded by the 20S proteasome *in vitro* in the absence of any ubiquitylation machinery.

NS2 protein

HCV NS2 protein is a transmembrane protein, composed of a highly hydrophobic N-terminal membrane binding domain and a C-terminal globular and cytosolic protease subdomain. NS2 protease cleaves off the N-terminus of NS3 protein and is involved in the assembly of HCV particles[57,58]. NS2 protein is also a short-lived protein that is rapidly degraded by the proteasome in a phosphorylation-dependent manner through the activity of casein kinase 2 (CK2). NS2 is phosphorylated by CK2 on a serine residue at position 168, which is a part of a consensus CK2 phosphorylation sequence motif (S/TXXE)[59]. This CK2 phosphoacceptor motif is highly conserved among NS2 proteins from all HCV genotypes. No ubiquitin conjugation of NS2 has been detected[59] and lysine mutagenesis has been reported to have no effect on NS2 levels[60]. These results suggest that the degradation of the HCV NS2 protein is ubiquitin-independent but proteasome-dependent.

HCV INFECTION-INDUCED UBIQUITIN-DEPENDENT DEGRADATION OF CELLULAR PROTEINS *VIA* THE PROTEASOME

Retinoblastoma tumor-suppressor protein

The abundance of the retinoblastoma tumor-suppressor protein (pRb) is negatively regulated in HCV RNA replicon cells[61] and HCVcc-infected cells[19]. The HCV RNA-dependent RNA polymerase NS5B protein forms a complex with pRb, targeting it for degradation, resulting in a reduction of pRb, the activation of the E2F-responsive promoter, and the promotion of cell proliferation[61]. NS5B contains a Leu-x-Cys/Asn-x-Asp motif that is homologous to the Rb-binding domains in the oncoproteins of DNA viruses and interacts with pRb through this motif. The ectopic expression of NS5B induces the polyubiquitylation of pRb, the abundance of which is restored by the siRNA knockdown of E6AP or by the overexpression of a dominant-negative E6AP mutant in HCV RNA replicon cells. This suggests the involvement of E6AP in pRb degradation, induced by HCV. However, it has been reported previously in an *in vitro* assay that the ubiquitylation of pRb is not promoted by E6AP, either in the presence or absence of NS5B[19]. The precise mechanism by which NS5B-dependent pRb ubiquitylation occurs thus remains to be clarified.

Suppressor of cytokine signaling 3

Suppressor of cytokine signaling 3 (SOCS3) is one of the negative regulators of cytokine signaling that function *via* the JAK-STAT pathway[62,63]. The SOCS3 protein levels have been found to be decreased in OR6 cells harboring a HCV genotype 1b replicon and also in Huh 7.5.1 cells infected with the HCV genotype 2a strain JFH1[64]. Treatment with the proteasome inhibitor MG132 blocked the inhibitory effects of HCV on the SOCS3 protein levels in both the replicon-harboring OR6 cells and JFH1-infected cells. JFH1 infection increased the ubiquitylation of SOCS3 compared with the mock infected cells. These results have suggested that HCV infection promotes the degradation of SOCS3 through the ubiquitin-dependent proteasome pathway. The underlying mechanism remains to be elucidated.

HCV INFECTION AFFECTS THE IMMUNOPROTEASOME

Proteasomal epitope processing

The induction of $CD8^+$ T cells is dependent on the generation of MHC class I ligands by the proteasome. Whereas the amino-terminus of each epitope can be further defined by post-proteasomal aminoexopeptidases, the carboxyl terminus needs to be defined precisely by the first cleavage. Through the study of a single source outbreak of HCV, Seifert *et al*[65] have previously identified a mutation at a conserved tyrosine on the HCV NS3 protein, which was a tyrosine to phenylalanine substitution. This mutation was found to impair the correct carboxyl-terminal cleavage of an immune-dominant, HLA-A2 restrictive HCV $NS3_{1073-1081}$ epitope from its mutated polypeptide precursor, not only by the constitutive proteasomes, but also by the immunoproteasome. These mutations impair the induction of HCV-specific $CD8^+$ T cells by affecting the proteasomal antigen-processing machinery.

MHC class I -restricted HCV antigen presentation and the effects of ethanol on this process

In Huh-7 cells co-expressing the HCV core protein and CYP2E1, the core protein slightly enhances 20S proteasome activity through a direct interaction and *via* the induction of low CYP2E1-dependent oxidative stress[66,67]. This proteasome activation event is, however, reversed after ethanol exposure which considerably reduces proteasome function due to the induction of high oxidative stress[66]. Ethanol-elicited suppression of the proteasome in the liver ultimately results in a reduced generation of

Table 1 The ubiquitin-dependent and -independent pathways that target hepatitis C virus proteins for degradation

HCV protein	E3 ligase	Function	Ref.
Ubiquitin-dependent, ATP-dependent, 26S proteasome pathway			
Core	E6AP[1]	Inhibit virus production	[14-17]
E2-p38	Unknown[1]	Immune avoidance	[47]
NS5A	Unknown[1]	Inhibit replication	[49]
NS5B	Unknown[1]	Inhibit replication	[50]
Ubiquitin-independent, ATP-independent, 20S proteasome pathway			
Core	PA28γ[2]	Enhance virus production, steatosis	[15,18,52,53]
F	-[2]	Unknown	[56]
NS2	CK2[2]	Unknown	[57,60]

[1]E3 ligase; [2]Host factor. HCV: Hepatitis C virus; CK2: Casein kinase 2.

antigenic peptides and reduced MHC class I -restricted antigen presentation on hepatocytes[68].

Low-molecular-mass protein 7

The HCV NS3 protein interacts with low-molecular-mass protein 7 (LMP7), a component of the immunoproteasome[69]. The minimal binding domain required for this interaction is located between the pro-sequence region of LMP7 (aa 1-40) and the protease domain of NS3. LMP7 has no effects on NS3 protease activity *in vitro*. The peptidase activities of LMP7 immunoproteasome, however, are markedly reduced in a HCV RNA subgenomic replicon. These findings suggest that the downregulation of proteasome peptidase activities could interfere with the processing of viral antigens for presentation by MHC class I molecules, thereby contributing to persistent infection by HCV.

CONCLUSION

In the present review, the current knowledge on the involvement of the ubiquitin-proteasome pathway and ubiquitin-independent proteasome pathway on HCV infection is summarized (Figure 1 and Table 1). As is the case with many other virus types, HCV may manipulate the ubiquitin system and the proteasome system to favor its propagation and contribute to viral pathogenesis. The body of knowledge regarding the ubiquitin-system and the proteasome system has markedly grown in recent years[21,22] and it has now been demonstrated that canonical Lys 48-linked polyubiquitin chains are not the only signals that initiate proteasome-mediated degradation. Monoubiquitylation[70], Lys 63-linked chains[71], Lys 11-linked chains[72], and linear chains[73-75] have been reported to have various functions, including the activation of signaling pathways and cell-cycle progression. Nothing is known however about the involvement of de-ubiquitylating enzymes in the HCV life cycle. The future identification of key molecules in the ubiquitin and proteasome systems will likely provide new insights and a better understanding of the life cycle and pathogenesis of HCV, knowledge which will be essential for the design of novel anti-HCV therapeutics.

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NS5A Sequence Heterogeneity of Hepatitis C Virus Genotype 4a Predicts Clinical Outcome of Pegylated-Interferon–Ribavirin Therapy in Egyptian Patients

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Hepatitis C virus genotype 4 (HCV-4) is the cause of approximately 20% of the 180 million cases of chronic hepatitis C in the world. HCV-4 infection is common in the Middle East and Africa, with an extraordinarily high prevalence in Egypt. Viral genetic polymorphisms, especially within core and NS5A regions, have been implicated in influencing the response to pegylated-interferon and ribavirin (PEG-IFN/RBV) combination therapy in HCV-1 infection. However, this has not been confirmed in HCV-4 infection. Here, we investigated the impact of heterogeneity of NS5A and core proteins of HCV-4, mostly subtype HCV-4a, on the clinical outcomes of 43 Egyptian patients treated with PEG-IFN/RBV. Sliding window analysis over the carboxy terminus of NS5A protein identified the IFN/RBV resistance-determining region (IRRDR) as the most prominent region associated with sustained virological response (SVR). Indeed, 21 (84%) of 25 patients with SVR, but only 5 (28%) of 18 patients with non-SVR, were infected with HCV having IRRDR with 4 or more mutations (IRRDR ≥ 4) ($P = 0.0004$). Multivariate analysis identified IRRDR ≥ 4 as an independent SVR predictor. The positive predictive value of IRRDR ≥ 4 for SVR was 81% (21/26; $P = 0.002$), while its negative predictive value for non-SVR was 76% (13/17; $P = 0.02$). On the other hand, there was no significant correlation between core protein polymorphisms, either at residue 70 or at residue 91, and treatment outcome. In conclusion, the present results demonstrate for the first time that IRRDR ≥ 4, a viral genetic heterogeneity, would be a useful predictive marker for SVR in HCV-4 infection when treated with PEG-IFN/RBV.

Hepatitis C virus (HCV) is a major cause of chronic liver disease, hepatocellular carcinoma, and deaths from liver disease and is the most common indication for liver transplantation (7, 26–28, 38). HCV has been classified into seven major genotypes and a series of subtypes (35, 36). In general, HCV genotype 4 (HCV-4) is common in the Middle East and Africa, where it is responsible for more than 80% of HCV infections (23). Although HCV-4 is the cause of approximately 20% of the 180 million cases of chronic hepatitis C in the world, it has not been a major subject of research.

Egypt has the highest prevalence of HCV worldwide (15%) and the highest prevalence of HCV-4, which is responsible for 90% of the total HCV infections, with a predominance of the subtype 4a (HCV-4a) (1, 32). This extraordinarily high prevalence results in an increasing incidence of hepatocellular carcinoma in Egypt, which is now the second most frequent cause of cancer and cancer mortality among men (17, 21). More than 2 decades have passed since the discovery of HCV, and yet therapeutic options remain limited. Up to 2011, the standard treatment for chronic hepatitis C consisted of pegylated alpha interferon (PEG-IFN) and ribavirin (RBV) (19); however, by May 2011 two protease inhibitors (telaprevir and boceprevir) were approved by the Food and Drug Administration (FDA) for use in combination with PEG-IFN/RBV for adult chronic hepatitis C patients with HCV genotype 1 (24, 34). Since the approval of these new protease inhibitors for treatment of HCV-1 infection, the response of HCV-4 to the standard regimen of treatment (PEG-IFN/RBV) has lagged behind other genotypes and HCV-4 has become the most resistant genotype to treat. As PEG-IFN/RBV still remains to be used to treat HCV-4-infected patients, exploring the factors that predict the outcome of PEG-IFN/RBV treatment, such as sustained virological response (SVR), for HCV-4 infections is needed to assess more accurately the likelihood of SVR and thus to make more informed treatment decisions.

While the SVR rate for PEG-IFN/RBV treatment hovers at 50 to 60% in HCV-1 and -4 infection, it is up to 80% in HCV-2 and -3 infections (19, 33). This difference in responses among patients infected with different HCV genotypes suggests that viral genetic heterogeneity could affect, at least to some extent, the sensitivity to IFN-based therapy. In this context, the correlation between IFN-based therapy outcome and sequence polymorphisms within the viral core and NS5A proteins has been widely discussed, in particular in regard to Japanese patients with HCV-1b infection. Initially, in the era of IFN monotherapy, it was proposed that sequence variations within a region in NS5A of HCV-1b, called the IFN sensitivity-determining region (ISDR), were correlated with IFN responsiveness (18). Subsequently, in the era of PEG-IFN/

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RBV combination therapy, we identified a new region near the C terminus of NS5A, referred to as the IFN/RBV resistance-determining region (IRRDR) (13). Recently, we also demonstrated the correlation between IRRDR polymorphism and PEG-IFN/RBV treatment outcome in HCV-2a and -2b infections (15). In addition, HCV core protein polymorphism, in particular at positions 70 and 91, was also proposed as a pretreatment predictor of poor virological response in patients infected with HCV-1b (4–6). To the best of our knowledge, there is no information regarding the correlation between sequence heterogeneity in the NS5A and core proteins of HCV-4 and PEG-IFN/RBV treatment outcome. In the present study, we aimed to investigate this issue in Egyptian patients infected with HCV-4.

MATERIALS AND METHODS

Ethics statement. The study protocol, which conforms to the provisions of the Declaration of Helsinki, was approved beforehand by the Ethic Committees in Cairo University Hospital and in Kobe University, and written informed consent was obtained from each patient prior to the treatment.

Patients. A total of 43 previously untreated patients who were chronically infected with HCV-4a (34 patients), HCV-4m (3 patients), HCV-4n (3 patients), or HCV-4o (3 patients) were consecutively evaluated for antiviral treatment at Cairo University Hospital, Cairo, Egypt, between January 2008 and September 2010. The HCV subtype was determined according to the method of Okamoto et al. (31). The patients were treated with PEG-IFN α-2a (180 μg/week, subcutaneously) and RBV (1,000 to 1,200 mg daily, *per os*) for 48 weeks. The quantification of serum HCV RNA titers was performed as previously reported (14). To minimize the therapeutic burdens, including the high cost and possible side effects, therapy was discontinued if HCV RNA titers at week 12 did not drop by 2 log compared with baseline values or if HCV RNA was still detectable at week 24. These were considered a null response (see Results).

Sequence analysis of the NS5A and core regions of the HCV genome. Blood samples were collected using Vacutainer tubes. The sera were separated within 2 h of blood collection, transferred to sterile cryovials, and kept frozen at −80°C until use. HCV RNA was extracted from 140 μl of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). The extracted RNA was reverse transcribed and amplified for the HCV genome encoding a carboxy terminus of NS5A (amino acids [aa] 2193 to 2417) and the core protein (aa 1 to 191) using SuperScript III one-step RT-PCR Platinum *Taq* HiFi (Invitrogen, Tokyo, Japan). The resultant reverse transcription (RT)-PCR product was subjected to a second-round PCR by using Platinum *Taq* DNA polymerase high fidelity III (Invitrogen). Primers used for amplification of the 3′ half of the NS5A region of HCV-4 were as follows: NS5A-4/F1 (5′-CTCAAYTCGTTCGT RGTGGGATC-3′; sense) and NS5A-4/R1 (5′-CGAAGGTCACCTTCTT CTGCCG-3′; antisense) for one-step RT-PCR; and NS5A-4/F2 (5′-ATG CGAGCCYGAGCCGGACGT-3′; sense) and NS5A-4/R2 (5′-GCTCAGG GGGYTRATTGGCAGCT-3′; antisense) for the second-round PCR. Primers for amplification of the core region of HCV-4 were 249-F (5′-G CTAGCCGAGTAGTGTTG-3′; sense) and 984-R (5′-GATGTGRTGRTC GGCCTC-3′; antisense) (40) for one-step RT-PCR; and 319-F (5′-GGA GGTCTCGTAGACCGTGC-3′; sense) (40) and primer-186 (5′-ATGTA CCCCATGAGGTCGGC-3′; antisense) (2) for the second-round PCR. RT was performed at 45°C for 30 min and terminated at 94°C for 2 min, followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 90 s. The second-round PCR was performed under the same conditions. The sequences of the amplified fragments were determined by direct sequencing without subcloning. The amino acid sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan). The numbering of amino acid residues for HCV-4 isolates is according to the polyprotein of ED43 isolate (accession no. Y11604) (10). Consensus sequences of the carboxy terminus of NS5A of a given HCV-4 subtype were inferred by alignment of all sequences obtained in this study as well as all available NS5A sequences of HCV-4a (accession no. Y11604, DQ418782 to DQ418789, DQ516084, and DQ988073 to DQ988079), HCV-4m (FJ462433), HCV-4n (FJ462441), and HCV-4o (FJ462440) from the databases.

TABLE 1 Virological responses of HCV-4-infected patients treated with PEG-IFN/RBV

Virological response	Proportion (%) of patients with indicated response (no. of patients/total no.)				
	HCV-4[a]	HCV-4a	HCV-4 m	HCV-4n	HCV-4o
SVR	58 (25/43)	56 (19/34)	100 (3/3)	33 (1/3)	67 (2/3)
Non-SVR	42 (18/43)	44 (15/34)	0 (0/3)	67 (2/3)	33 (1/3)
Null response	30 (13/43)	32 (11/34)	0 (0/3)	67 (2/3)	0 (0/3)
Relapse	12 (5/43)	12 (4/34)	0 (0/3)	0 (0/3)	33 (1/3)

[a] Includes all 43 cases with HCV-4 infection (34 cases with HCV-4a and 3 cases each with HCV-4m, -4n, and -4o).

Statistical analysis. Numerical data were analyzed by Student's *t* test and categorical data by Fisher's exact probability test. To evaluate the optimal threshold of the number of amino acid mutations in IRRDR for prediction of treatment outcomes, the receiver operating characteristic (ROC) curve was constructed. Univariate and multivariate logistic regression analyses were performed to identify independent predictors for treatment outcomes. All statistical analyses were performed using the SPSS version 16 software (SPSS Inc., Chicago, IL). Unless otherwise stated, a P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB725987 through AB726066.

RESULTS

Patients' responses to PEG-IFN/RBV combination therapy. Among 43 patients enrolled in this study, 30 (70%) patients completed the entire course of PEG-IFN/RBV treatment for 48 weeks and follow-up for 24 weeks. On the other hand, the treatment was discontinued for 13 (30%) patients due to poor virological responses at 12 or 24 weeks after initiation of the therapy. Overall, 25 (58%) patients achieved SVR while 18 (42%) patients had non-SVR (Table 1). When analyzed on the basis of the subtype classification, SVR was achieved by 56% (19/34), 100% (3/3), 33% (1/3), and 67% (2/3) of patients infected with HCV-4a, -4m, -4n, and -4o, respectively.

Non-SVR patients are classified into two groups: (i) patients with null response, who did not achieve >2-log reduction of the initial viral load at week 12 or who had detectable viremia at week 24 of the treatment period; and (ii) patients with relapse, who were negative for HCV-RNA at the end of the treatment period (week 48) followed by a rebound viremia at a certain time point during the follow-up period of 24 weeks. Patients with null response represented 30% (13/43) of all the HCV-4-infected subjects analyzed, while those with relapse represented 12% (5/43). A similar tendency was observed for subtype HCV-4a.

Among various patients' demographic characteristics, SVR patients had a significantly lower average age than that of non-SVR patients (Table 2). Furthermore, a tendency for SVR patients to have a lower average titer of initial viral load than that of non-SVR was noted, although the difference was not statistically significant, due possibly to the small number of patients analyzed ($P = 0.07$).

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TABLE 2 Demographic characteristics of HCV-4-infected patients with SVR and non-SVR[a]

Factor	SVR	Non-SVR	*P* value
Age	38.47 ± 9.51	45.80 ± 5.65	0.014
Sex (male/female)	18/7	15/3	0.48
BMI	27.36 ± 3.65	27.67 ± 5.28	0.85
Platelets (× $10^3/\mu l$)	204.4 ± 40.63	216.7 ± 87.25	0.59
Hemoglobin (g/dl)	14.54 ± 1.38	15.08 ± 1.39	0.25
WBC count	7,041 ± 1,876	7,078 ± 2,977	0.96
Albumin (g/dl)	4.12 ± 0.36	4.328 ± 0.41	0.11
ALT (IU/liter)	78.72 ± 59.68	82.39 ± 41.80	0.83
AST (IU/liter)	64.94 ± 27.63	58.17 ± 23.98	0.44
HCV-RNA (IU/ml)	84,290 ± 186,300	501,800 ± 816,700	0.07

[a] Values are means ± standard deviations. SVR, sustained virological response; BMI, body mass index; WBC, white blood cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Correlation between NS5A sequence heterogeneity and SVR in HCV-4 infection. We and other researchers reported significant correlation between sequence polymorphisms within the C-terminal half of NS5A, including that in ISDR and IRRDR, and PEG-IFN/RBV treatment outcome in HCV-1 and HCV-2 infections (13, 15, 18, 30). However, this information is quite limited in HCV-4 infection. To clarify this issue, part of the HCV-4 genome encoding a carboxy terminus (aa 2193 to 2417) of NS5A in pretreatment sera was amplified and sequenced, and amino acid sequences were deduced. The sequences obtained as well as all available NS5A sequences of HCV-4a, -4m, -4n, and -4o from the databases were aligned, and the consensus sequences for a desired HCV-4 subtype were inferred (see Materials and Methods). Next, to identify an NS5A region(s) that would be significantly correlated with treatment outcome, we carried out a sliding window analysis with a window size of 30 residues over the C-terminal half (aa 2193 to 2417) of NS5A sequences obtained from all SVR (n = 25) and non-SVR (n = 18) patients along with corresponding consensus sequences of each HCV-4 subtype as described previously (30). This analysis revealed that the difference in the overall number of amino acid mutations between SVR and non-SVR isolates exceeded the significant threshold only in a region corresponding to IRRDR of HCV-1b (13), ranging from aa 2331 to 2383, thus being referred to as IRRDR[HCV-4] (Fig. 1). Indeed, the average number of amino acid mutations in IRRDR[HCV-4] was significantly larger in SVR than in non-SVR (P = 0.0005) isolates (Fig. 2A). Sequences of IRRDR of HCV-4a, -4m, -4n, and -4o obtained from SVR and non-SVR patients along with the number of IRRDR mutations of each isolate are shown in Fig. 2B.

Next, we performed ROC curve analysis to estimate the optimal cutoff number of IRRDR[HCV-4] mutations for SVR prediction. This analysis estimated 4 mutations as the optimal number of IRRDR[HCV-4] mutations to predict SVR, since it achieved the highest sensitivity (84%; sensitivity refers to the proportion of SVR patients who were infected with HCV isolates of IRRDR[HCV-4] with 4 or more mutations) and specificity (72%; specificity refers to the proportion of non-SVR patients who were infected with HCV isolates of IRRDR[HCV-4] with 3 or fewer mutations) with an area under the curve (AUC) of 0.82 (Fig. 3). Accordingly, 21 (84%) of 25 patients with SVR, in contrast to only 5 (28%) of 18 patients with non-SVR, had IRRDR[HCV-4] with 4 or more mutations (referred to as IRRDR[HCV-4]≥4), with the difference between the two groups being statistically significant (P = 0.0004) (Table 3). It should be noted that 4 (31%) of 13 patients with null response and only 1 (20%) of 5 patients with relapse had HCV with IRRDR[HCV-4]≥4. These results collectively suggest that IRRDR[HCV-4]≥4 is significantly associated with SVR. In this connection, we also tested the impact of a higher (≥5) and a lower (≥3) degree of IRRDR mutations on treatment outcome. IRRDR[HCV-4]≥5 was significantly associated with SVR, though with a relatively lower sensitivity (64%) than that of IRRDR[HCV-4]≥4 (Table 3). On the other hand, there was no significant correlation between IRRDR[HCV-4]≥3 and SVR.

Correlation between core protein sequence heterogeneity and SVR in HCV-4 infection. A close correlation between core protein sequence patterns at positions 70 and 91 and treatment outcome has been proposed, especially in Japanese patients with HCV-1b infection (4–6). To examine this hypothesis in Egyptian patients infected with HCV-4, core sequences of the viral genome were amplified from the pretreated sera, and the amino acid sequences were deduced. Due to a high degree of sequence homology among core sequences of various HCV-4 subtypes, all sequences obtained were aligned with the prototype sequence, ED43 (10). The residues at positions 70 and 91 were both well conserved among the sequences analyzed, and therefore, no correlation with treatment outcome was observed for these residues (Fig. 4). All but two isolates had arginine at position 70 (Arg^{70}), the residue that has been associated with an IFN-sensitive phenotype as far as the core protein of HCV-1b is concerned (4–6). On the other hand, Pro at position 71 showed a tendency to be more frequent in SVR than in non-SVR patients; however, the frequency was not statistically different between the two groups.

Identification of independent predictive factors for SVR in HCV-4 infection. In order to identify significant independent

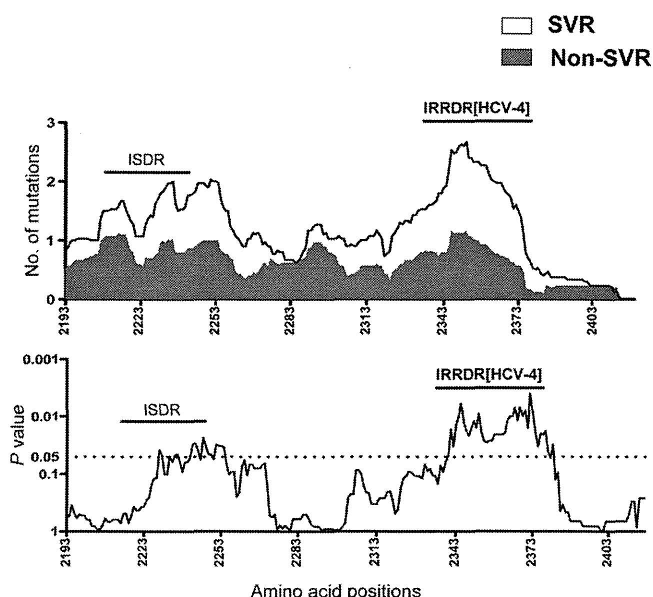

FIG 1 Sliding window analysis over the carboxy terminus (aa 2193 to 2417) of NS5A of HCV-4 obtained from SVR and non-SVR patients.

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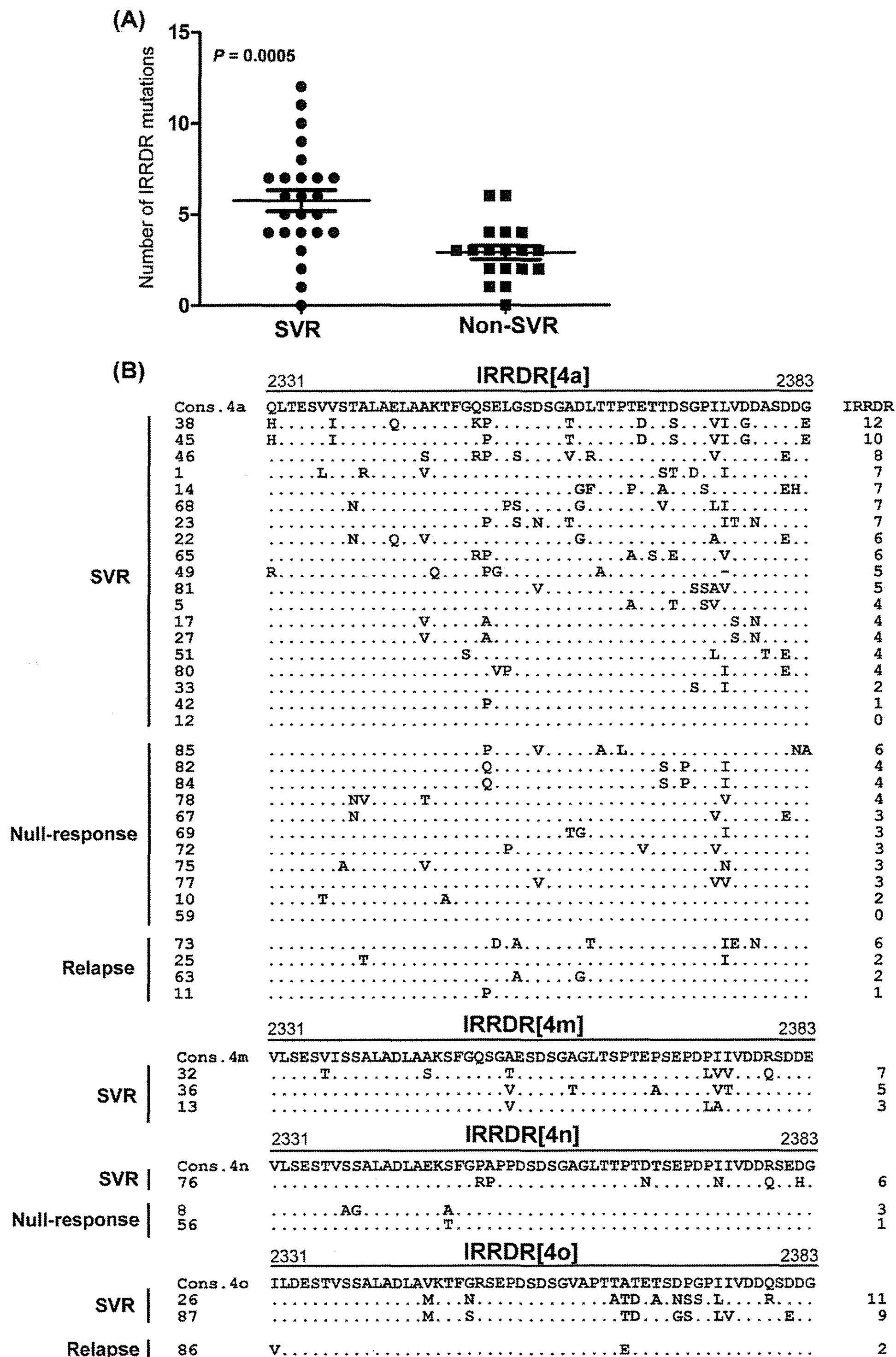

FIG 2 Correlation between IRRDR[HCV-4] sequence variations and treatment outcome. (A) Average number of amino acid mutations in IRRDR[HCV-4] obtained from SVR and non-SVR patients. (B) Alignment of IRRDR[HCV-4] sequences obtained from SVR and non-SVR patients with HCV-4a, -4m, -4n, and -4o. The consensus sequence (Cons) of each subtype is shown on the top. The numbers along the sequence indicate the amino acid positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in each IRRDR (4a, 4m, 4n, or 4o) are shown on the right.

predictive factors of SVR for PEG-IFN/RBV treatment outcome in HCV-4 infection, first, all available data of baseline patients' parameters and IRRDR[HCV-4] polymorphism were entered in a univariate logistic analysis. This analysis yielded 3 factors that were correlated or nearly correlated with SVR: IRRDR[HCV-4]≥4 ($P = 0.0004$), patient's age (<42 years; $P = 0.03$), and HCV RNA titer (<5,200 IU/ml; $P = 0.08$). Subsequently, these 3 factors were entered in multivariate logistic regression analysis. This anal-

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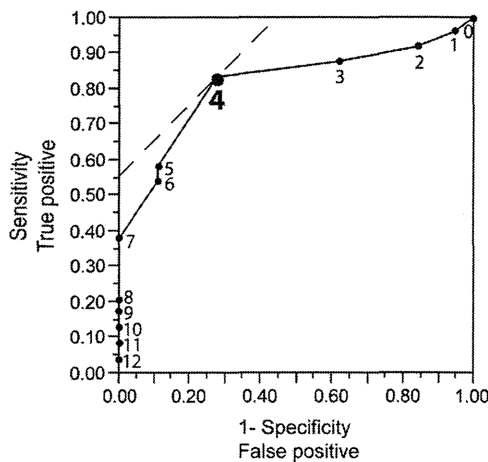

FIG 3 ROC curve analysis of IRRDR[HCV-4] sequence heterogeneity for SVR prediction. The solid line curve shows the AUC. Solid circles with numerals plotted on the curve represent different numbers of IRRDR mutations analyzed. The dashed line in the upper left corner indicates the optimal number of IRRDR[HCV-4] mutations for SVR prediction, which yields the highest sensitivity (84%) and the highest specificity (72%).

ysis revealed that the IRRDR[HCV-4]≥4 was the only independent predictive factor for SVR in HCV-4 infection (Table 4). We then assessed SVR predictability by means of IRRDR[HCV-4]≥4. As shown in Table 5, IRRDR[HCV-4]≥4 would predict SVR with a positive predictive value (PPV) of 81% (P = 0.002) and sensitivity of 84%. On the other hand, IRRDR[HCV-4]≤3 would predict non-SVR with a negative predictive value (NPV) of 76% (P = 0.02) and specificity of 72%. Thus, the degree of sequence variation in IRRDR[HCV-4] would yield useful positive and negative predictive markers for PEG-IFN/RBV therapy outcome in HCV-4-infected patients.

DISCUSSION

Both host and viral genetic factors have been implicated in influencing the clinical response to PEG-IFN/RBV therapy for HCV infection (22). It has recently been reported that host genetic polymorphisms near or within the IL28B gene on chromosome 19 show a critical impact on the treatment outcome of patients infected with HCV-1 (20, 37, 39). As for the viral factor(s), polymorphisms of NS5A and core regions of a given HCV genotype have been linked to a difference in SVR rates (3, 4, 13, 18, 30). This hypothesis was mostly inferred from studies carried out with Asian populations, in particular Japanese, with HCV-1b infection. However, whether it can be applied to non-Asian populations infected with non-HCV-1 is still unknown. To the best of our knowledge, this is the first study that specifically examines the relationship between HCV genome heterogeneity, in particular in NS5A and core regions, and PEG-IFN/RBV treatment outcome in Egyptian patients infected with HCV-4. In analogy with our previous studies that identified IRRDR as a significant determinant for PEG-IFN/RBV treatment outcome in Japanese patients infected with HCV-1b, -2a, and -2b (12–16), we have demonstrated in the present study that sequence heterogeneity within IRRDR is closely associated with the ultimate treatment outcome in Egyptian patients infected with HCV-4. A high degree of sequence variation in IRRDR[HCV-4], i.e., more than 4 (IRRDR ≥ 4), significantly correlated with SVR, while a low degree of sequence variation in this region (IRRDR ≤ 3) correlated with non-SVR, null response, and relapse. The majority of patients with SVR (84%) had HCV with IRRDR of ≥4. In contrast, nearly two-thirds (72%) of the patients with non-SVR had HCV with IRRDR ≤ 3 (P = 0.0004) (Table 3). Notably, 21 of the 26 patients infected with HCV with IRRDR[HCV-4]≥4 achieved SVR. Accordingly, the PPV and NPV of IRRDR[HCV-4]≥4 for SVR and non-SVR patients were 81% (P = 0.002) and 76% (P = 0.02), respectively (Table 5). Our present results thus strongly suggest that the degree of sequence heterogeneity within IRRDR[HCV-4] would be a useful marker for prediction of treatment outcome in HCV-4 infection.

The molecular mechanism underlying the possible involvement of this region in IFN responsiveness of the virus is still unknown. The significant difference among IRRDR sequence patterns may suggest genetic flexibility of this region. Indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions (29). This flexibility might play an important role in modulating the interaction with various host systems, including IFN-induced antiviral machineries. It is also possible that the genetic flexibility of IRRDR is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN-based therapy (8, 29, 41). Also, it is worth noting that IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV (25) whereas its upstream and downstream sequences show a higher degree of sequence conservation (15). This may suggest that whereas the upstream and downstream sequences have a conserved function(s) across all the HCV genotypes, IRRDR sequences have a genotype-dependent or even a strain-dependent function(s).

A mutation at position 70 of the core protein of HCV-1b has been reported to be correlated with PEG-IFN/RBV treatment out-

TABLE 3 Correlation between NS5A sequence heterogeneity and virological responses in HCV-4 infection

	No. of isolates/total no. (%)				P value for SVR versus:		
Factor	SVR	Non-SVR	Null response	Relapse	Non-SVR	Null response	Relapse
IRRDR ≥ 4	21/25 (84)[a]	5/18 (28)	4/13 (31)	1/5 (20)	0.0004	0.003	0.01
IRRDR ≤ 3	4/25 (16)	13/18 (72)[b]	9/13 (69)	4/5 (80)			
IRRDR ≥ 5	16/25 (64)[a]	2/18 (11)	1/13 (8)	1/5 (20)	0.0006	0.002	0.14
IRRDR ≤ 4	9/25 (36)	16/18 (89)[b]	12/13 (92)	4/5 (80)			
IRRDR ≥ 3	22/25 (88)[a]	11/18 (61)	10/13 (77)	1/5 (20)	0.066	0.39	0.006
IRRDR ≤ 2	3/25 (12)	7/18 (39)[b]	3/13 (23)	4/5 (80)			

[a] Sensitivity (proportion of SVR patients with the favorable factor).
[b] Specificity (proportion of non-SVR patients with the unfavorable factor).

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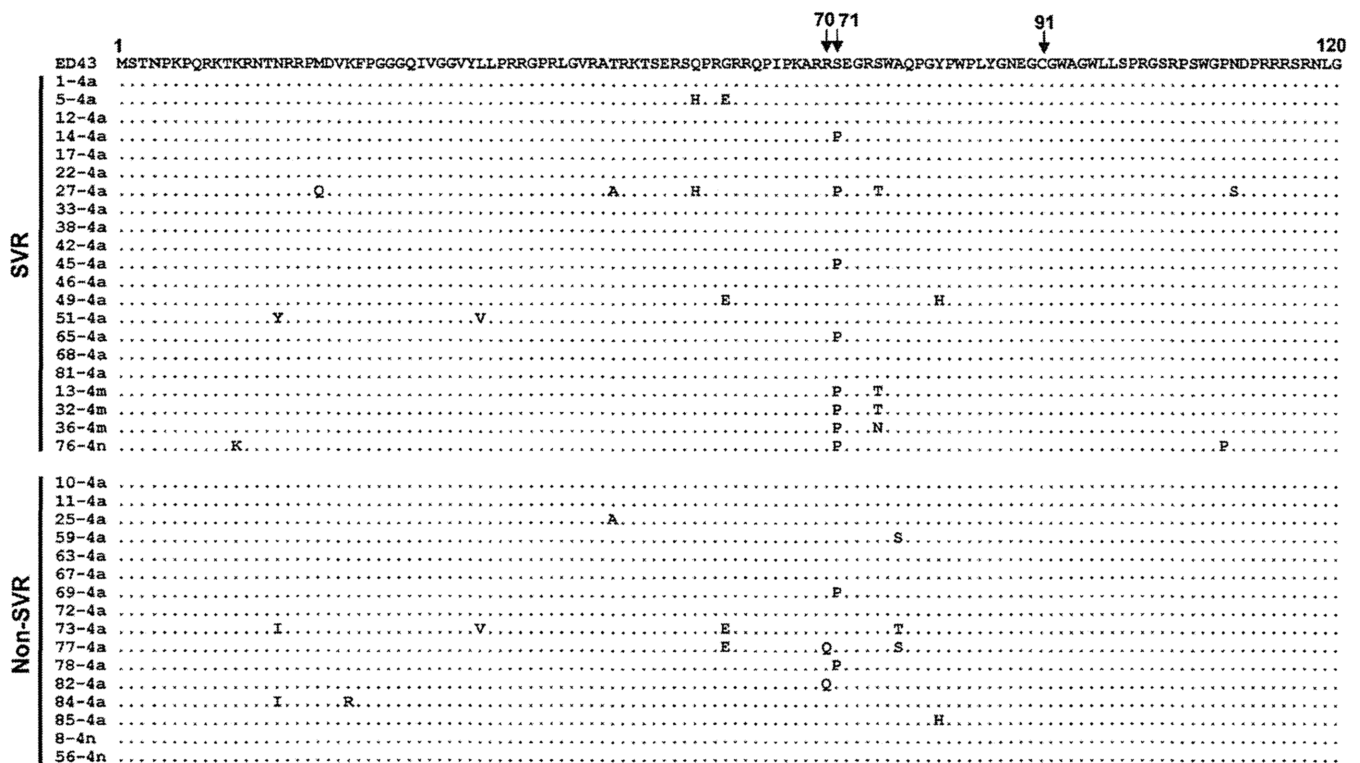

FIG 4 Sequence alignment of the core protein of HCV-4 isolates. Core protein sequences (aa 1 to 120) of HCV-4 obtained from SVR and non-SVR patients are aligned. The prototype sequence of ED43 (10) is shown on the top. The numbers along the sequence indicate the amino acid positions. Dots indicate residues identical to those of the prototype sequence.

come (4, 12). In the present study, however, we found no significant correlation between core protein polymorphism and treatment outcome in HCV-4 infection. The residue at position 70 of the core protein of all but two HCV-4 isolates analyzed in this study was Arg (Fig. 4), which is known to be associated with SVR in HCV-1b infection (4, 12). This high degree of sequence conservation at position 70 might be the reason for the lack of significant correlation between core protein polymorphism and treatment outcome in HCV-4 infection.

Single nucleotide polymorphisms (SNPs) near the IL28B region have been identified as the strongest baseline predictors of SVR to PEG-IFN/RBV in patients with HCV-1 infection. More recently, in two major studies that were carried out exclusively with HCV-4-infected patients (9, 11), the CC genotype of rs12979860 IL28B SNP was also strongly associated with SVR. It is worth noting that although the SVR rate was more than 80% among the patients with the CC genotype, these patients represented only around 40% of total SVR cases in both studies. Furthermore, the CC genotype was found in only 34% of all Egyptian patients analyzed (9). Taken together, those observations support the idea that in addition to IL28B polymorphism, there should be an additional factor(s) that influences SVR. In this context, an interplay between IRRDR and IL28B polymorphisms might explain why some patients with undesirable IL28B genotype achieve SVR and why some patients infected with HCV isolates with IRRDR[HCV-4]≥4 do not achieve SVR. Further comprehensive study is needed to validate the importance of IRRDR and IL28B polymorphisms in predicting the treatment outcome of HCV-4-infected patients.

In conclusion, the present study emphasizes the importance of IRRDR sequence heterogeneity in the prediction of PEG-IFN/RBV treatment outcome for different HCV genotype infections in

TABLE 4 Univariate and multivariate analyses for identification of independent predictive factors for SVR in HCV-4-infected patients treated with PEG-IFN/RBV therapy

Univariate analysis		Multivariate analysis	
Variable	*P* value	Odds ratio (95% CI)	*P* value
IRRDR mutations (IRRDR ≥ 4 versus IRRDR ≤ 3)	0.0004	10.5 (1.12–98.91)	0.04
Age (<42 years)	0.03		
HCV-RNA (<5,200 IU/ml)	0.08		

TABLE 5 PPV, NPV, sensitivity, and specificity of IRRDR sequence heterogeneity on the likelihood of achieving SVR and non-SVR in HCV-4 infection

Factor	PPV	NPV	Sensitivity[c]	Specificity[d]
IRRDR ≥ 4	81% (21/26)[a]		84% (21/25)	
IRRDR ≤ 3		76% (13/17)[b]		72% (13/18)

[a] $P = 0.002$.
[b] $P = 0.02$.
[c] Proportion of SVR patients who were infected with HCV isolates with IRRDR of ≥4.
[d] Proportion of non-SVR patients who were infected with HCV isolates with IRRDR of ≤3.

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different ethnic groups, including Egyptian patients infected with HCV-4.

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No conflicts of interest exist.

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Role of the Endoplasmic Reticulum-associated Degradation (ERAD) Pathway in Degradation of Hepatitis C Virus Envelope Proteins and Production of Virus Particles*[S]

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Background: HCV causes ER stress in the infected cells.
Results: HCV-induced ER stress leads to increased expression of certain proteins that in turn enhance the degradation of HCV glycoproteins and decrease production of virus particles.
Conclusion: HCV infection activates the ERAD pathway, leading to modulation of virus production.
Significance: ERAD plays a crucial role in the viral life cycle.

Viral infections frequently cause endoplasmic reticulum (ER) stress in host cells leading to stimulation of the ER-associated degradation (ERAD) pathway, which subsequently targets unassembled glycoproteins for ubiquitylation and proteasomal degradation. However, the role of the ERAD pathway in the viral life cycle is poorly defined. In this paper, we demonstrate that hepatitis C virus (HCV) infection activates the ERAD pathway, which in turn controls the fate of viral glycoproteins and modulates virus production. ERAD proteins, such as EDEM1 and EDEM3, were found to increase ubiquitylation of HCV envelope proteins via direct physical interaction. Knocking down of EDEM1 and EDEM3 increased the half-life of HCV E2, as well as virus production, whereas exogenous expression of these proteins reduced the production of infectious virus particles. Further investigation revealed that only EDEM1 and EDEM3 bind with SEL1L, an ER membrane adaptor protein involved in translocation of ERAD substrates from the ER to the cytoplasm. When HCV-infected cells were treated with kifunensine, a potent inhibitor of the ERAD pathway, the half-life of HCV E2 increased and so did virus production. Kifunensine inhibited the binding of EDEM1 and EDEM3 with SEL1L, thus blocking the ubiquitylation of HCV E2 protein. Chemical inhibition of the ERAD pathway neither affected production of the Japanese encephalitis virus (JEV) nor stability of the JEV envelope protein. A co-immunoprecipitation assay showed that EDEM orthologs do not bind with JEV envelope protein. These findings highlight the crucial role of the ERAD pathway in the life cycle of specific viruses.

Quality control of proteins, such as the elimination of misfolded proteins, is largely connected with the endoplasmic reticulum (ER),[2] which is an organelle responsible for the folding and distribution of secretory proteins to their sites of action. This pathway is termed ER-associated degradation (ERAD) and is triggered by ER stress. It results in retrotranslocation of misfolded proteins into the cytosol, followed by polyubiquitylation and proteasomal degradation (1). Several viral infections have been reported to trigger the ERAD pathway (2–4); however, the role of this pathway in the life cycle of viruses remains poorly defined.

Initiation of the ERAD pathway occurs from the oligomerization and autophosphorylation of IRE1, an ER stress sensor. The activated IRE1 removes an intron from X-box-binding protein 1 (XBP1) mRNA, which then encodes a potent transcription factor for activation of genes, for example, ER degradation-enhancing α-mannosidase-like protein (EDEM). EDEM1 (5), along with its two homologs EDEM2 (6) and EDEM3 (7), as well as ER mannosidase I (ER ManI), belong to the glycoside hydrolase 47 family. EDEMs are thought to function as lectins that deliver misfolded glycoproteins to the ERAD pathway. However, the precise mechanism by which they assist in glycoprotein quality control remains unclear.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease. The RNA genome of HCV, a member of the Fla-

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[2] The abbreviations used are: ER, endoplasmic reticulum; CHX, cycloheximide; EDEM, ER degradation-enhancing α-mannosidase-like protein; ERAD, ER-associated degradation; HCV, hepatitis C virus; JEV, Japanese encephalitis virus; KIF, kifunensine; ManI, mannosidase I; m.o.i., multiplicity of infection; TM, tunicamycin; XBP1, X-box-binding protein 1; IRE, inositol-requiring enzyme.

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viridae family, encodes the viral structural proteins Core, E1, E2, and p7, as well as six nonstructural proteins (8, 9). Two *N*-glycosylated envelope proteins E1 and E2 are exposed on the surface of the virus and are necessary for viral entry.

The aim of this study was to investigate whether the ERAD pathway is activated upon HCV infection and whether this affects the quality control of virus glycoproteins and virion production. We show that HCV infection triggers the ERAD pathway, possibly through IRE1-mediated splicing of XBP1. Moreover, EDEM1 and EDEM3, but not EDEM2, interact with HCV glycoproteins, resulting in increased ubiquitylation. EDEM1 knockdown and chemical inhibition of the ERAD pathway increases glycoprotein stability, as well as production of infectious virus particles, whereas overexpression of EDEM1 decreases virion production. These results provide insight into the mechanism by which HCV triggers the ERAD pathway and subsequently affects the quality control of virus glycoproteins and virus particle production.

EXPERIMENTAL PROCEDURES

Cell Culture and Chemicals—Human hepatoma cells HuH-7 and HuH-7.5.1 (a gift from Dr. F. V. Chisari (The Scripps Research Institute) (10) and human embryonic kidney cells 293T were cultured at 37 °C and 5% CO_2 in DMEM containing 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, nonessential minimum amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. Tunicamycin (TM) was purchased from Sigma-Aldrich, and kifunensine (KIF) was purchased from Toronto Research Chemicals (Ontario, Canada).

Preparation of Virus Stock—HCV JFH-1 was generated by introducing *in vitro* transcribed RNA into HuH-7.5.1 cells by electroporation, and virus stocks were prepared by infecting at a multiplicity of infection (m.o.i.) of 0.01, as described previously (10). Infected cells were grown in culture medium containing 2% FBS, and supernatants were collected after multiple passages to get high titer virus. The supernatants were concentrated using a 500-kDa hollow fiber module (GE Healthcare) resulting in ~90% recovery of the virus. Focus-forming units were measured with an anti-HCV core antibody to determine virus titration (2H9, described below). Virus stocks containing 1×10^7 focus-forming units/ml were divided into small aliquots and stored at −80 °C until use. rAT strain of Japanese encephalitis virus (JEV) (11) was used to generate virus stock.

Plasmids—cDNAs of mouse EDEM1-HA, EDEM2, and EDEM3-HA, having 92, 93, and 91% amino acid homology with their human orthologs, respectively, were a kind gift from Drs. N. Hosokawa (Kyoto University) and K. Nagata (Kyoto Sangyo University). A HA tag was attached to the C terminus of EDEM2 by PCR, and sequencing analysis was performed to confirm the sequence. To generate pJFH/E1dTM-myc and pJFH/E2dTM-myc, HCV E1 encoding amino acids 170–352 and HCV E2 encoding amino acids 340–714 of JFH-1 polyprotein were amplified by PCR with forward primer and reverse primer containing NotI and XbaI restriction sites, respectively, and cloned into a NotI/XbaI site of the pEF1/Myc-His plasmid (Invitrogen). The pCAGC105E plasmid carrying PrM and E proteins of the rAT strain of JEV has been described (12). Plasmids carrying the firefly luciferase reporter gene under control of the intact promoter of GRP78 and GRP94 or the defective promoter lacking ERSE elements have been described (13) and were a kind gift from Dr. K. Mori (Kyoto University).

Antibodies—Rabbit polyclonal antibodies included anti-HA (Sigma-Aldrich), anti-HCV NS5A (14), anti-SEL1L (Sigma-Aldrich), anti-ubiquitin (MBL, Nagoya, Japan), and anti-JEV E antibodies. The mouse monoclonal antibodies were anti-HA (clone 16B12; Covance, Emeryville, CA), anti-HCV E2 (clone 8D10-3),[3] anti-β-actin (clone AC15; Sigma-Aldrich), anti-HCV core (clone 2H9) (15), and anti-Myc (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Anti-JEV antibodies have been described (16) and were a kind gift from Drs. C. K. Lim and T. Takasaki (National Institute of Infectious Diseases).

Analysis of XBP1 Splicing—Total RNA was extracted from cells using Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol, and 2 μg of RNA was subjected to cDNA synthesis using oligo(dT) and Superscript III (Invitrogen). PCR was carried out using specific primers 5′-AAACAGAGTAGCAGCTCAGACTGC-3′ and 5′-GTATCTCTAAGACTAGGGGCTTGGTA-3′ for XBP1 and 5′-TCCTGTGGCATCCACGAAACT-3′ and 5′-GAAGCATTTGCGGTGGACGAT-3′ for β-actin to generate PCR fragments of 598 bp for unspliced XBP1, 572 bp for spliced XBP1, and 315 bp for β-actin. The following cycling conditions were used to amplify the genes: 1 cycle of 98 °C for 3 min, followed by 30 cycles of 98 °C for 20 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. The PCR product of XBP1 was further digested with PstI enzyme (New England Biolabs) and resolved on a 2% agarose gel prepared in TAE buffer. Unspliced XBP1 yielded two smaller fragments of 291 and 307 bp whereas spliced XBP1 stayed intact due to loss of the restriction site after splicing.

Gene Microarray Analysis—For microarray analysis, RNA was extracted from HuH-7.5.1 cells at 48 and 72 h after JFH-1 infection. Cells treated for 12 h with 5 μg/ml TM served as a positive control. Hybridization was performed on a 3D-Gene (see 3D-Gene web site) Human Oligonucleotide chip 25k (Toray Industries Inc., Tokyo, Japan). For efficient hybridization, this microarray chip has three dimensions and is constructed with a well between the probes and cylinder stems with 70-mer oligonucleotide probes on the top. Total RNA was labeled with Cy3 or Cy5 using the Amino Allyl MessageAMP II aRNA Amplification kit (Applied Biosystems). The Cy3- or Cy5-labeled aRNA pools were subjected to hybridization for 16 h using the supplier's protocol. Hybridization signals were scanned using a ScanArray Express Scanner (PerkinElmer Life Sciences) and processed by GenePixPro version 5.0 (Molecular Devices, Sunnyvale, CA). Detected signals for each gene were normalized using a global normalization method (Cy3/Cy5 ratio median = 1). Genes with Cy3/Cy5 normalized ratios $>\log_2 1.0$ or $<\log_2 -1.0$ were defined, respectively, as significantly up- or down-regulated genes.

Quantification of Cellular Gene Expression—Gene expression levels were measured using predesigned assay-on-demand (Applied Biosystems). RT-PCR amplification was performed

[3] D. Akazawa, N. Nakamura, and T. Wakita, unpublished data.

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under the following conditions: 48 °C for 30 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. Standard curves were constructed on a 1:5 serial dilution of the RNA template. The results were normalized to GAPDH mRNA levels.

Determination of Protein Stability—HuH-7 cells were infected with HCV JFH-1 at a m.o.i. of 2. Six hours after infection, the cells were either treated with KIF or transfected with EDEM1 siRNA. Forty hours later, culture medium was replaced with 100 μg/ml cycloheximide (CHX). Cells, including floating cells, were harvested at different time points after CHX addition, and immunoblotting was performed to determine the amount of HCV E2.

Plasmid Transfection and Immunoprecipitation—HuH-7 or 293T cells were seeded in 6-well cell culture plates at 3×10^5 cells/well and cultured overnight. Plasmid DNA was transfected into cells using TranIT-LT1 transfection reagent (Mirus, Madison, WI). Cells were harvested at 48 h after transfection, washed once with 1 ml of PBS, and lysed in 200 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 135 mM NaCl, 1% Triton X-100, and 10% glycerol supplemented with 50 mM NaF, 5 mM Na_3VO_4, and protease inhibitor mixture tablets (Roche Diagnostics). Cell lysates were sonicated at 4 °C for 10 min, incubated for 30 min at 4 °C, and centrifuged at 14,000 × *g* for 5 min at 4 °C. After preclearing for 2 h, the supernatants were immunoprecipitated overnight by rotating with 1.5 μl of anti-HA monoclonal antibody (16B12) or anti-HCV E2 monoclonal antibody (clone 8D10-3) at 4 °C. The immunocomplexes were then captured on protein G-agarose beads (Invitrogen) by rotation-incubation at 4 °C for 3 h. Beads were subsequently precipitated by centrifugation at 800 × *g* for 1 min and washed five times with lysis buffer. Finally, proteins bound to the beads were boiled in 40 μl of SDS sample buffer and subjected to SDS-PAGE.

Western Blotting—Proteins resolved by SDS-PAGE were transferred onto PVDF membranes (Immobilon; Millipore). After blocking in 2% skim milk, the membranes were probed with primary antibodies followed by exposure to peroxidase-conjugated secondary antibodies and visualization with an ECL Plus Western blotting detection system (GE Healthcare). The intensity of the bands was measured using a computerized imaging system (ImageJ software; National Institutes of Health).

Small Interfering RNA (siRNA) Transfection—HuH-7 cells were transfected with duplex siRNAs at a final concentration of 10 nM using Lipofectamine RNAiMAX (Invitrogen). Three siRNAs for each gene were examined for knock-down efficiency and cytotoxic effects. The siRNA with best performance was selected for further experiments. Target sequences of the siRNAs which exhibited the best knock-down efficiencies were as follows: EDEM1 (sense) 5′-CAUAUCCUCGGGUGAAUCUtt-3′, EDEM2 (sense) 5′-GAAUGUCUCAGAAUUCCAAtt-3′, EDEM3 (sense) 5′-CAUGAGACUACAAAUCUUAtt-3′, IRE1 (sense) 5′-GGACGUGAGCGACAGAAUAtt-3′. 5′-GGUGUCCUUACCAUACUAAtt-3′ served as a negative control. The lowercase letters denote overhanging deoxyribonucleotides.

Quantification of HCV Core and RNA—HCV core was quantified using an enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics, Tokyo, Japan). HCV RNA was quantified as described (17).

Statistical Analysis—Student's *t* test was employed to calculate the statistical significance of the results. $p < 0.05$ was considered significant.

RESULTS

HCV Infection Induces XBP1 mRNA Splicing and EDEM Expression—XBP1 plays a key role in activating the ERAD pathway, which mediates unfolded protein response in the ER. Under conditions of ER stress, XBP1 mRNA is processed by unconventional splicing and translated into functional XBP1, which in turn mediates transcriptional up-regulation of a variety of ER stress-dependent genes. The resultant activation of downstream pathways boosts the efficiency of ERAD, which coincides with elevated transcription of EDEMs. To validate our method for detecting activation of the ERAD pathway, we exposed HuH-7.5.1 cells to TM, which is a typical ER stress inducer, and performed an assay to quantify spliced XBP1 mRNA, as described under "Experimental Procedures," at different time points after treatment. The spliced form of XBP1 mRNA started accumulating within these cells as early as 2 h after exposure to TM (Fig. 1*A*), and levels remained elevated until at least 12 h after treatment. Quantitative RT-PCR showed that mRNA levels of EDEM1, EDEM2, and EDEM3 were elevated in TM-treated cells whereas ER ManI, which is not an ER stress-responsive gene, did not show any up-regulation (Fig. 1*B*). To examine involvement of the ERAD pathway in the HCV life cycle, we infected HuH-7.5.1 cells with JFH-1 at m.o.i. of 5 and analyzed XBP1 mRNA splicing and EDEM up-regulation. Upon infection, the fragment corresponding to spliced XBP1 mRNA, was detectable 8 h after infection, and the difference in splicing between mock- and HCV-infected cells became more pronounced at 48 h after infection and then persisted (Fig. 1*C*). Increased levels of XBP1 mRNA splicing were dependent on the m.o.i. (supplemental Fig. 1*A*), suggesting that expression of active XBP1 was induced by HCV infection. A small amount of spliced XBP1 was detected in mock-infected cells, presumably because of some intrinsic stress. A 3.1-fold increase in the level of EDEM1 mRNA was observed at 3–4 days after infection ($p < 0.05$). Increases in EDEM2 and EDEM3 mRNA levels were moderate and reached ~1.5-fold, whereas ER ManI mRNA exhibited no change after infection (Fig. 1*D*). Expression of EDEMs, particularly EDEM1, was up-regulated in accordance with HCV infection titers (supplemental Fig. 1*B*). Knocking down the IRE1 gene (Fig. 1*E*) effectively reversed the accumulation of spliced XBP1, as well as the transcriptional up-regulation of EDEM1 (Fig. 1*F*), thus confirming that HCV infection induces ERAD through the IRE1-XBP1 pathway.

To enable a comprehensive investigation of the transcriptional changes that occur, up- and down-regulation of the transcriptome was examined in HCV-infected cells and in TM-treated cells. The results were compared with those of mock-transfected cells at each time point. A range of genes involved in ER stress was found to be regulated in HCV-infected and in TM-treated cells (Fig. 2*A*). EDEM1 was signifi-

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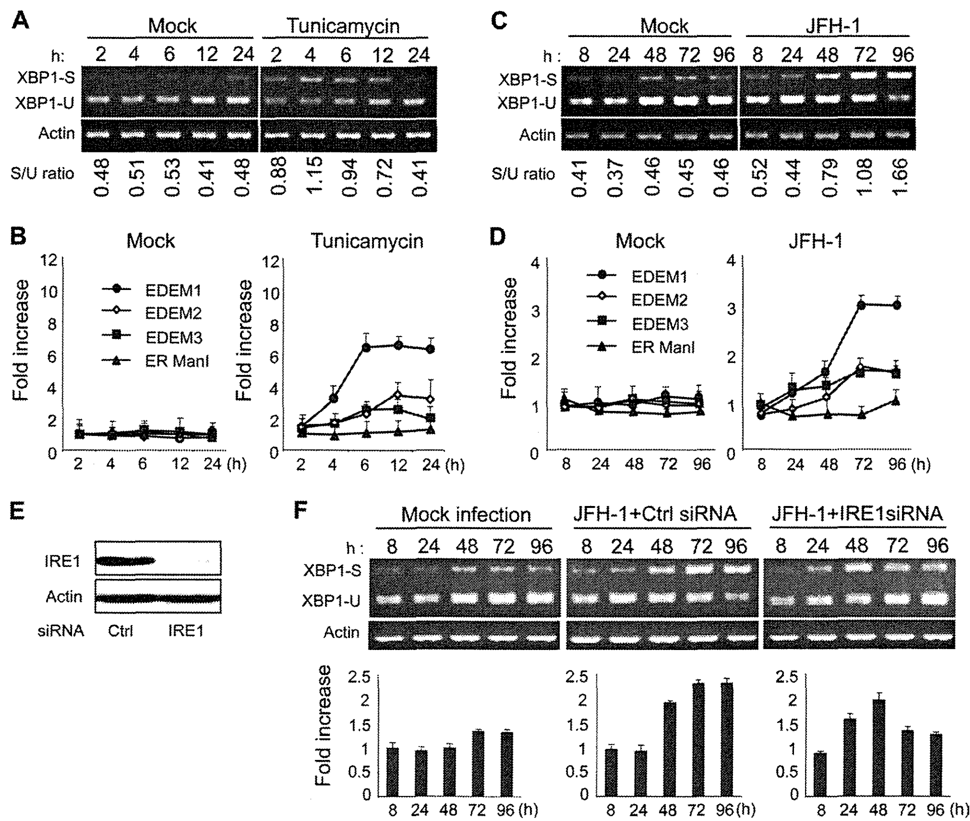

FIGURE 1. **Splicing of XBP1 mRNA and induction of ERAD gene expression in HCV JFH-1-infected cells.** *A*, splicing of XBP1 mRNA analyzed in mock- and TM (5 μg/ml)-treated HuH-7.5.1 cells at different time points after treatment. The *upper* and *lower bands* represent spliced and unspliced RNA, respectively. The *numbers* at the *bottom* of the *panel* indicate the density ratios of bands corresponding to spliced and unspliced XBP1. *B*, graphs showing the -fold induction of EDEM1, EDEM2, EDEM3, and ER Man1 mRNA in HuH-7.5.1 cells treated or untreated with TM. Data are normalized to GAPDH expression levels. The mean ± S.D. (*error bars*) of three independent experiments are shown. *C*, splicing of XBP1 mRNA analyzed in mock- and HCV JFH-1-infected HuH-7.5.1 cells (m.o.i. 5) at different time points after infection. *Numbers* at the *bottom* of the *panel* indicate the density ratios of bands corresponding to spliced and unspliced XBP1. *D*, real-time PCR analysis of EDEM1, EDEM2, EDEM3, and ER ManI mRNA induction in mock- and HCV-infected cells. Data are normalized to GAPDH expression. The mean ± S.D. of three independent experiments are shown. Note that a reduction in the level of GAPDH mRNA within infected cells was not observed until 96 h after infection when a slight decrease was observed. This led us to use GAPDH as a housekeeping gene in our experiments. *E*, Western blotting of IRE1 in cells transfected with mock or gene-specific siRNA of IRE1. *F*, splicing of XBP1 mRNA and induction of EDEM1 in HCV-infected cells after knocking down of the IRE1 gene. HuH-7.5.1 cells infected with JFH-1 at a m.o.i. of 5 were transfected with mock (*center*) or IRE1-specific siRNA (*right*) 48 h after infection, after which splicing of XBP1 (*upper*) and transcriptional up-regulation of EDEM1 (*lower*) were examined at the indicated time points after infection. The mean ± S.D. of two independent experiments are shown.

cantly up-regulated upon HCV infection, whereas expression levels of EDEM2 and EDEM3 remained unchanged. Although transcriptional changes caused by HCV infection in many of the genes listed are analogous to those that occur in cells treated with TM, up-regulation of two ER chaperone proteins, GRP78 and GRP94, was induced by TM treatment but not by HCV infection. This differential induction was confirmed by a reporter assay for GRP78 promoter and GRP94 promoter activities (Fig. 2*B*). These results are in agreement with a previously described finding that GRP78 and GRP94 are not responsive to HCV infection in hepatoma cells (18). It remains likely that HCV infection interferes with transcriptional activation of some ER chaperone proteins; however, the mechanism by which this occurs remains to be elucidated.

EDEMs Cause Ubiquitylation of HCV Glycoproteins and Enhance Their Degradation—Because EDEMs have been reported to enhance proteasomal degradation of ERAD substrates through direct binding, we investigated the interaction of EDEMs with HCV glycoproteins in 293T cells by co-transfecting the expression plasmids for E1dTM or E2dTM together with plasmids carrying either EDEM or ER ManI genes. Immunoprecipitation and immunoblotting demonstrated that each EDEM, but not ER ManI, was capable of interacting with E2 (Fig. 3*A*) and E1 (supplemental Fig. S2). HCV glycoproteins displayed enhanced mobility when co-expressed with EDEM1, EDEM3, or ER ManI, which could be due to the mannosidase activity of these proteins, which is lacking in EDEM2 (6). HCV primarily replicates in hepatocytes so we examined the interaction of EDEMs with E2dTM in HuH-7 cells as well, which yielded similar results (data not shown). E2dTM lacks the transmembrane domain, which could affect its folding and ER retention and thus modulate the ability of this protein to interact with EDEMs and ER ManI. Second, E1 and E2 glycoproteins assemble as noncovalent heterodimers to make functional complexes, which may alter the interaction of these proteins with EDEMs. To address these issues, we co-transfected HuH-7 cells with plasmids carrying full-length E1E2 glycoproteins together with plasmids carrying either EDEMs or ER ManI. Similar phenotypes were produced following transfection full-length E1E2 proteins (supplemental Fig. S3*A*), demonstrating that functional complexes of HCV glycoproteins bind with EDEMs. Recently, we have reported on the development of a

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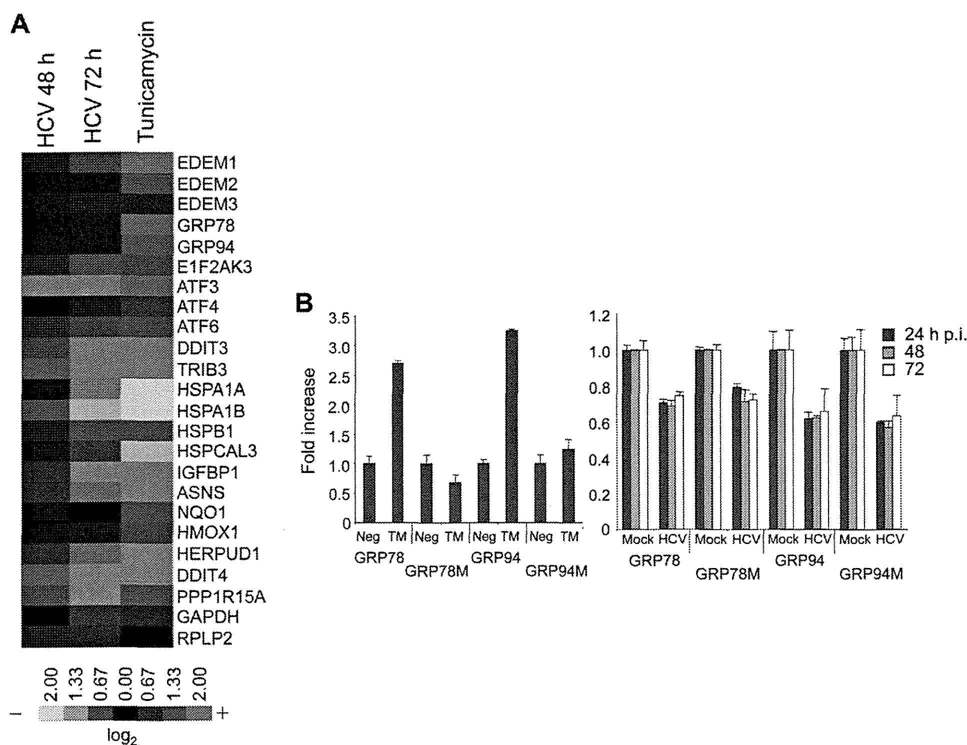

FIGURE 2. **Comprehensive analysis of ERAD gene expression in JFH-1-infected HuH-7.5.1 cells.** *A*, HuH-7.5.1 cells treated with TM (5 μg/ml) for 12 h or infected with JFH-1 for 48 and 72 h were subjected to microarray analysis, along with their negative controls. Expression of ER stress genes is shown as a heat map. *Red* and *green* indicate up- and down-regulation, respectively. Information on each gene shown is indicated on the 3D-Gene web site. *B*, GRP78 and GRP94 induction in TM-treated (*left*) and HCV-infected cells (*right*). GRP78M and GRP94M represent the defective promoters. The mean ± S.D. (*error bars*) of three independent experiments are shown.

packaging system of HCV subgenomic replicon sequences through the provision of viral core NS2 proteins in *trans* (19). Transcomplementation with core NS2 proteins resulted in successful packaging of the viral sequences; therefore, plasmids carrying these proteins are a valid construct by which to examine the interaction of envelope proteins with ERAD machinery. Thus, we performed an immunoprecipitation assay of HuH-7 cells co-transfected with core NS2 and EDEMs. In agreement with our previous results, EDEMs, but not ER ManI, were observed to bind to HCV E2 protein (supplemental Fig. S3*B*). To examine the functional importance of this interaction, we analyzed the ubiquitylation of HCV E2 protein in cells co-transfected with HCV E2 and EDEM proteins. An immunoprecipitation assay revealed that overexpression of EDEM1 and EDEM3, but not of EDEM2 and ER ManI, dramatically increased the ubiquitylation of HCV glycoprotein (Fig. 3*B*). In mammals, the ER membrane ubiquitin-ligase complex involved in the dislocation of ERAD substrates, and their ubiquitylation contains the ER membrane adaptor SEL1L. It has recently been shown that SEL1L interacts with EDEM1 in cells and functions as a cargo receptor for ERAD substrates (20); however, it is unknown whether SEL1L interacts with other EDEMs. We therefore assessed whether SEL1L interacts with EDEM1, EDEM2, EDEM3, and ER ManI in cells (Fig. 3*C*). Interestingly, endogenous SEL1L co-precipitated with EDEM1 and EDEM3, whereas little to no interaction was observed with EDEM2 and ER ManI. Collectively, it is likely that, although all EDEMs can recognize HCV E1 and E2, EDEM1 and EDEM3 are involved in the ubiquitylation of HCV glycoproteins by delivering them to SEL1L-containing ubiquitin-ligase complexes. To investigate further the role of EDEMs in quality control of HCV glycoproteins, we measured the steady-state level of HCV E2 protein after EDEM knockdown. Transfection of HCV-infected cells with siRNAs against EDEM1, EDEM2, or EDEM3 caused a 60–80% reduction in mRNA levels of the respective genes (Fig. 3*D*) with no cytotoxic effects observed (data not shown). Immunoblotting showed a considerable increase in the steady-state level of viral E2 in EDEM1 siRNA-treated cells (Fig. 3*D*). We subsequently examined the turnover of E2 in cells with and without EDEM1 knockdown. In CHX half-life experiments, E2 protein was moderately unstable in control-infected cells, presumably via proteasomal degradation (Fig. 3*E*). Treatment with MG132, a proteasome inhibitor, blocked its destabilization (data not shown). In contrast, E2 was completely stable in EDEM1-knockdown cells during the chase period of time tested (Fig. 3*E*). Together, these results strongly suggest that EDEM1 and EDEM3, particularly EDEM1, are involved in the post-translational control of HCV glycoproteins.

Involvement of EDEM1 in the Production of Infectious HCV—Given the involvement of EDEMs in the turnover of HCV glycoproteins, we investigated whether EDEMs affect the replication and production of infectious virus particles. EDEMs were knocked down in HCV-infected HuH-7 cells by siRNA transfection, and the production of infectious particles was then monitored by measuring the extracellular infectivity titer. Knocking down of EDEM1 and EDEM3 in the infected cells resulted in ~3.1-fold ($p < 0.05$) and ~2.3-fold increases in virus production, respectively, compared with control cells. No effect

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