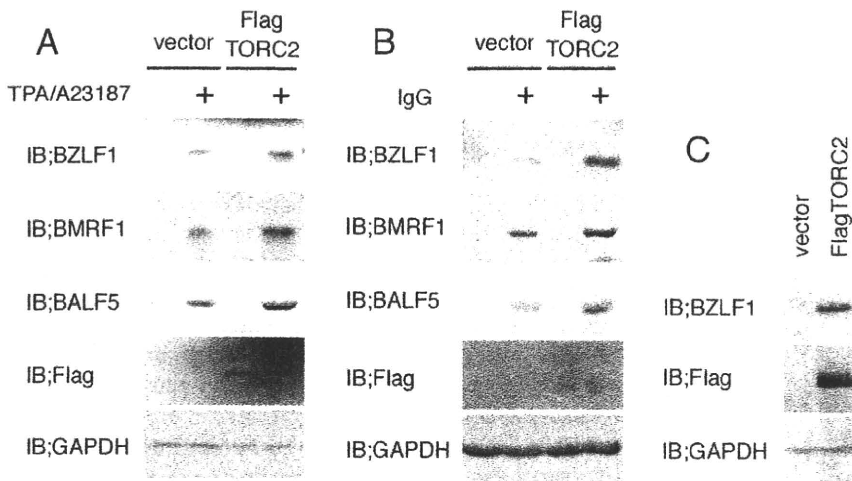

FIGURE 4. **Increased expression of the BZLF1 protein on exogenous expression of TORC2.** *A* and *B*, GTC-4 (*A*) cells or Akata (*B*) cells were transfected with the TORC2 expression vector, and 24 h thereafter, TPA(20 ng/ml) and A23187 (0.5 μM) or IgG was added to the culture followed by incubation for another 24 h and IB with anti-BZLF1, -BMRF1, -BALF5, -FLAG, and -GAPDH antibodies. *C*, EBV-293 cells transfected with the TORC2 expression vector were incubated for 24 h followed by IB with anti-BZLF1, -FLAG, and -GAPDH antibodies.

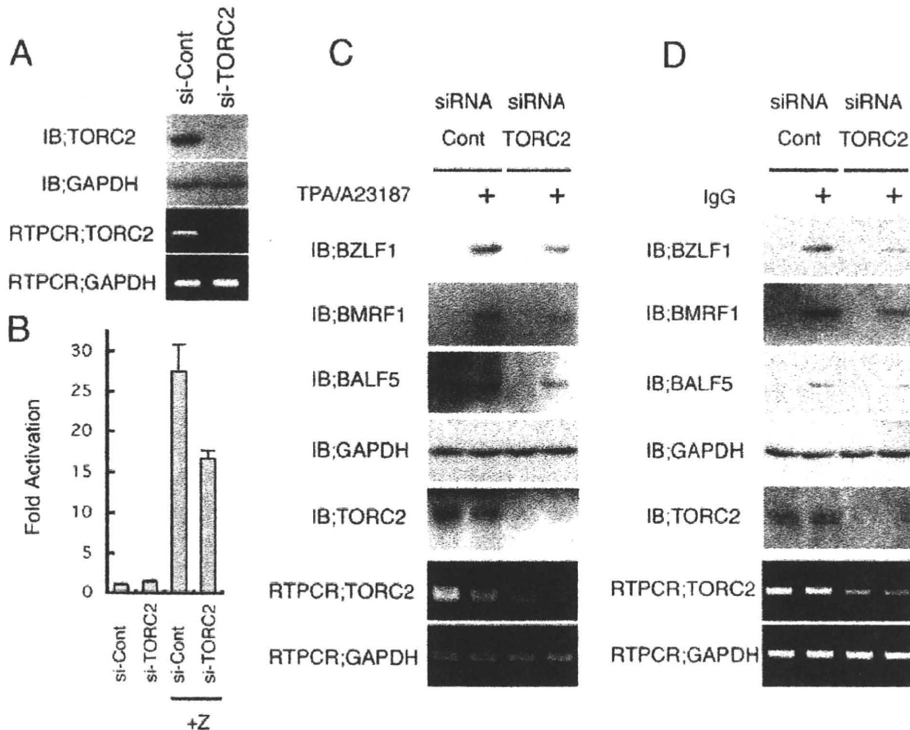

FIGURE 5. **BZLF1-mediated transcription depends on endogenous TORC2 expression.** *A* and *B*, HEK293T cells were transfected with duplexes of 21-nucleotide siRNA against TORC2 (*si-TORC2*) or control (*si-Cont*) siRNA together with 10 ng of pZp-luc with or without 10 ng pcDNABZLF1 (*+Z*). IB and RT-PCR assays (*A*) and luciferase assays (*B*) were carried out as described under "Experimental Procedures." The luciferase activity is shown as -fold activation of that with control siRNA without BZLF1. Each *bar* represents the mean and S.D. of three independent transfections. *C* and *D*, knock-down of TORC2 mRNA in GTC-4 (*C*) and Akata (*D*) cells. Cells transfected with siRNA against TORC2 (*siRNA TORC2*) or the Control (*siRNA Cont*) were cultured with TPA (20 ng/ml) and A23187 (0.5 μM) or IgG for 24 h. Protein or mRNA levels of BZLF1, BMRF1, BALF5, TORC2, and GAPDH were examined by IB or RT-PCR.

suggest a cooperative influence of the BZLF1 protein and dephosphorylated TORC2 in the presence of calcineurin signaling activation.

Role of TORC2 in EBV Reactivation from Latency—To examine the role of TORC2 in EBV reactivation from latency, GTC-4 cells, in which EBV is latently infected, were transfected with the TORC2 expression vector and incubated with or without TPA/A23187 (Fig. 4*A*). The cells expressed the BZLF1, BMRF1, and BALF5 proteins in response to TPA/A23187 treatment, and further exogenous expression of TORC2 increased the levels of the proteins. A similar result was obtained in Akata cells (Fig. 4*B*). Expression of S171A mutant of TORC2 appears to impact on BZLF1 levels significantly (data not shown). We also tested EBV-293 cells, in which levels of exogenous gene expression are very efficient (Fig. 4*C*). Even in the absence of TPA/A23187, overexpression of TORC2 clearly enhanced BZLF1 protein levels.

To examine the function of TORC2 under physiological conditions, we employed siRNA technology using a synthetic oligonucleotide that forms a duplex RNA encoding partial nucleotides from TORC2. As shown in Fig. 5*A*, treatment with siRNA against TORC2 reduced the level of TORC2 mRNA in HEK293T cells, whereas the level of GAPDH remained unchanged. TORC2 siRNA treatment also resulted in a decrease in the BZLF1-mediated transcription (Fig. 5*B*; *+Z, si-TORC2*) when compared with control siRNA treatment (*+Z, si-Cont*).

In addition, the effect of siRNA against TORC2 was also examined in GTC-4 and Akata cells, as shown in Fig. 5, *C* and *D*, respectively. Treatment with TORC2 siRNA suppressed the mRNA expression of TORC2, whereas the GAPDH gene was unaffected. The treatment also reduced the levels of viral lytic proteins including BZLF1.

To eliminate the possibility that the siRNA against TORC2 might elicit interferon signaling pathway, we analyzed interferon-β expression by RT-PCR (33) because activation of the signaling pathway provoked by double-stranded RNA causes the promoter activation. Treatment with TORC2 siRNA did not induce the levels of interferon-β (supplemental Fig. S2), indicating that interferon signaling is not activated by si-TORC2.

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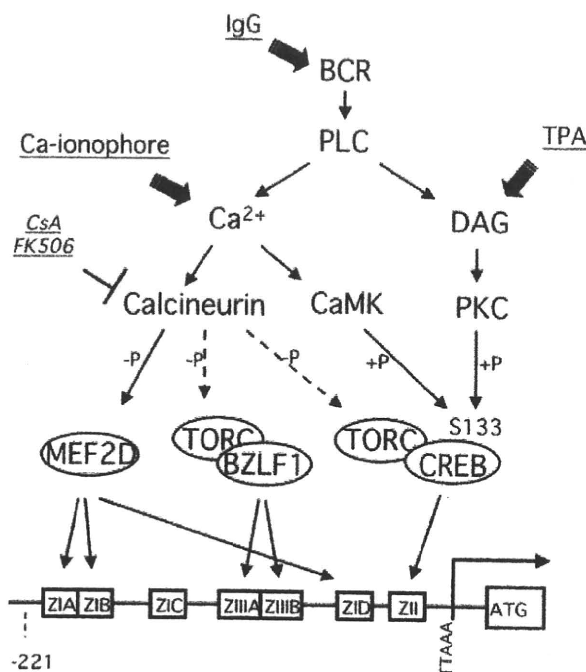

FIGURE 6. **Proposed model for EBV Zp activation.** TORC2, as well as myocyte enhancer factor 2D (*MEF2D*), is activated by calcineurin, a phosphatase that can be inhibited by CsA or FK506. TORC2 is able to associate with CREB or the BZLF1 protein and enhances Zp activity through binding to ZII and ZIII *cis* elements. *PLC*, phospholipase C; *PKC*, protein kinase C; *DAG*, diacylglycerol; *CaM*, calmodulin; BCR, B-cell receptor.

To deny the possibility that the TORC2 siRNA used in Fig. 5 might act through unknown off-target effects, we tested another TORC2 siRNA in supplemental Fig. S3. It also suppressed the expression level of the BZLF1 protein.

To test if not only TORC2 but also other TORCs might be involved in this process, all the members of TORC proteins were silenced simultaneously. In that case, however, reduction of the BZLF1 level was no stronger than that by si-TORC2 only (Fig. supplemental S4), suggesting the dominant role of TORC2 in this transcriptional activation. These results strongly suggest the importance of TORC2 in BZLF1 production and EBV reactivation from latency.

DISCUSSION

In this report we document evidence that TORC is able to enhance transcription from Zp and, more interestingly, that TORC interacts with the BZLF1 protein to activate the promoter very strongly. Fig. 6 shows our working model for Zp induction. Previous studies have demonstrated that both ZI and ZII elements are necessary for the initial activation (2, 34). It has been reported that myocyte enhancer factor 2D plays a crucial role in virus reactivation from latency (5), being dephosphorylated by calcineurin and enhancing its binding to ZI. CREB family transcription factors bind to ZII when phosphorylated by protein kinase C, calmodulin kinase, or possibly mitogen-activated protein kinases. In addition to the activation by phosphorylation, our reporter assays indicated that CREB is activated by TORC in a CREB phosphorylation-independent manner. Furthermore, our study strongly suggests that TORC also potentiates the promoter activity by binding to the ZIII element through the BZLF1 protein. Calcineurin, a serine/threonine-phosphatase sensitive to cyclosporin A is responsible for the dephosphorylation and the activation of TORC. In turn, cyclosporin A and FK506 are very effective for suppressing EBV.

A number of cellular proteins have been reported to interact with the BZLF1 protein, including p53 (35, 36), C/EBPα (37), NF-κB (38), basic transcriptional machinery TFII components (39), and CREB-binding protein (CBP) (40, 41). Among these, CBP has histone acetyltransferase activity and cooperates with the BZLF1 protein to transactivate BZLF1-dependent transcription, inducing the viral lytic cycle. Mutation analysis revealed that at least the homodimerization domain (b-Zip) of the BZLF1 protein is required for its interaction with CBP, but other parts of the protein also must be involved in the association (40, 41). The BZLF1 protein also interacts with TFII components mainly through the transactivation domain and stabilizes the association of initiation complexes on DNA. Stable assembly of general transcriptional machinery might promote transcription from BZLF1-responsive promoters. Interestingly, TORC enhances the interaction of CREB with the $TAF_{II}130$ component of TFIID (11), and at least TORC2 mediates target gene activation by associating with CBP/p300 and increasing its recruitment to CREB-responsive promoters (31). From these studies and our own results, the BZLF1 protein may not only directly recruit CBP/p300 and basic transcriptional machinery but also be able to recruit them through TORC2.

Besides EBV, transcription from human T-cell leukemia virus type 1 long terminal repeats is also affected by TORC proteins (21, 42). TORC activates long terminal repeats through interaction with the viral transcriptional factor Tax as well as CREB. So this mode of the action is quite parallel to the situation with EBV. Because both human T-cell leukemia virus type 1 and EBV are lymphotropic viruses, there is a possibility that other lymphotropic viruses such as the human immunodeficiency virus might also be controlled by TORC proteins.

Curiously, although CMV immediate-early promoter has a CREB binding motif (26), we here observe that transcription from the promoter is less affected by TORC proteins (Fig. 1*C*) when compared with the BZLF1 promoter, an EBV immediate-early gene. Others also have used expression vectors driven by the CMV immediate-early promoter and shown that the promoter activity is relatively unaffected (22, 42). It is speculated that this might be because the activation by TORC is dependent on the promoter context (11, 43). Because immediate-early genes of herpesviruses are crucial for lytic infection, distinct dependence of the promoters on TORC proteins may reflect differences in the characters of those herpesviruses.

Although TORC proteins could enhance Zp 100-fold in reporter assays, overexpression or ablation of TORC2 had only a relatively small impact on BZLF1 production under physiological conditions. It is likely that transcriptional suppressors of the promoter such as YY1 (44) might inhibit transcription. Another intriguing possibility is that there might be epigenetic regulation such as DNA methylation or histone deacetylation. Interestingly, Gruffat *et al.* (45) reported that myocyte enhancer factor 2 family protein, a crucial transactivator for the

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Zp, recruits class II histone deacetylases to suppress transcription from Zp. They argued that the switch from latency to the productive cycle is dependent at least in part on the post-translational modification of myocyte enhancer factor 2 and local acetylation state of histones around the Zp. Likewise, the transcriptional co-activator TORC can associate with BCL-3, which recruits histone deacetylases to inhibit transcription (22). These results and the cited reports suggest that the molecular mechanism regulating EBV reactivation from latency is not quite as simple as expected, and further clarification of the mechanism of BZLF1-mediated transcription is necessary. Elucidation of associating factors and chromosomal environment of the Zp proximity may contribute to the development of anti-EBV compounds.

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Identification of cellular and viral factors related to anti-hepatitis C virus activity of cyclophilin inhibitor

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We have so far reported that an immunosuppressant cyclosporin A (CsA), a well-known cyclophilin (CyP) inhibitor (CPI), strongly suppressed hepatitis C virus (HCV) replication in cell culture, and that CyPB was a cellular cofactor for viral replication. To further investigate antiviral mechanisms of CPI, we here developed cells carrying CsA-resistant HCV replicons, by culturing the HCV subgenomic replicon cells for 4 weeks in the presence of CsA with G418. Transfection of total RNA from the isolated CsA-resistant cells to naïve Huh7 cells conferred CsA resistance, suggesting that the replicon RNA itself was responsible for the resistant phenotype. Of the identified amino acid mutations, D320E in NS5A conferred the CsA resistance. The replicon carrying the D320E mutation was sensitive to interferon-α, but was resistant to CsA and other CPIs including NIM811 and sanglifehrin A. Knockdown of individual CyP subtypes revealed CyP40, in addition to CyPA and CyPB, contributed to viral replication, and CsA-resistant replicons acquired independence from CyPA for efficient replication. These data provide important evidence on the mechanisms underlying the regulation of HCV replication by CyP and for designing novel and specific anti-HCV strategies with CPIs. (*Cancer Sci* 2009; 100: 1943–1950)

Hepatitis C virus (HCV) is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC), and affects an estimated 170 million people worldwide.[1] The current standard therapy for patients infected with HCV is the combination treatment with pegylated interferon and ribavirin.[2,3] However, approximately half of individuals infected with HCV are unable to reach sustained virological response following such treatment. In addition, several side effects have been reported, which hinder continued treatment and impair the regimen efficacy. Thus, the development of novel anti-HCV strategies is essential for the treatment of infected individuals.

We have previously reported that a well-known immunosuppressant cyclosporin A (CsA) strongly suppressed the replication of HCV *in vitro*, in a manner independent of the interferon (IFN) signal transduction pathway.[4] Cyclophilin B (CyPB), a cellular target of CsA, was subsequently revealed to facilitate viral replication via the regulation of the RNA binding ability of NS5B.[5] Thus CyP, in addition to viral proteins including NS3 protease and NS5B polymerase, can also prove useful as a molecular target for antiviral strategies. Indeed, the non-immunosuppressive CsA analogs NIM811, DEBIO-025, and SCY635 have been observed to exert strong inhibitory effects on HCV replication, and these compounds are now in clinical trial.[6–8] Thus, it is crucial to deepen understanding of the anti-HCV actions of cyclophilin inhibitor (CPI) in order to maximize the efficacy of the agent. CPIs also need to face challenges such as side effects and drug resistance, which was observed as barrier to successful treatment in cases of human immunodeficiency virus (HIV),[9–12] and further clarification of the mechanism of CPI's anti-HCV activities is vital for the development of stronger and more specific therapeutic drug types. For this purpose, we here established and characterized the resistant replicon to CPIs using the subgenomic replicon system. We found that D320E, a mutation in NS5A, conferred resistance to CsA on the replicon, while additional mutations in NS3, Q86R and I252T seen in our CsA-resistant clone affected the replication fitness positively and negatively, respectively. The CsA-resistant replicons with the D320E mutation showed cross-resistance to other CPIs, NIM811 and sanglifehrin A (SFA), which were thus verified to suppress HCV replication through targeting CyP, and those resistant replicons were inhibited by treatment with IFNα as effectively as the wild type. Knockdown of individual CyP subtypes in the wild-type and CsA-resistant replicon cells revealed that CyP40, besides CyPA and CyPB, played important roles in HCV replication, and CyPA was related to the CsA-resistance. These results are important for elucidating additional mechanisms of the regulation of HCV replication by CyP and also for designing novel and specific anti-HCV strategies with CPI.

Materials and Methods

Compounds. CsA and IFNα were purchased from Merck Biosciences (San Diego, CA, USA) and Otsuka Pharmaceutical (Tokyo, Japan), respectively. NIM811 and SFA were generously provided by Novartis (Basel, Switzerland).

Cell culture. MH14 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, nonessential amino acids (Invitrogen), and L-glutamine (Invitrogen) in the presence of 700 μg/mL G418 (Invitrogen).

Establishment of cell clones. We established each cell clone along with the outline shown in Figure 1. CsR#4, CsR#10, and CsR#11 cells were established through the selection of MH14#12 cell colonies in the presence of 1000 μg/mL G418 and 2 μg/mL CsA. CsR#11-2 and CsR#11-3 cells were established from Huh7 cells transfected with total RNAs extracted from CsR#11 cells in the presence of 700 μg/mL G418. Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E cells were produced by 700 μg/mL G418 selection of Huh7 cells transfected with 5 μg RNA transcribed from pMH14 carrying the individual mutations Q86R in NS3 and D320E in NS5A, double mutations Q86R in NS3 and D320E in NS5A, and triple mutations Q86R in NS3, I252T in NS3, and D320E in NS5A, respectively.

Colony formation assay. MH14 cells were treated with either CsA or NIM811 in the presence of 700 μg/mL G418 for 4 weeks, followed by fixation and staining with crystal violet.

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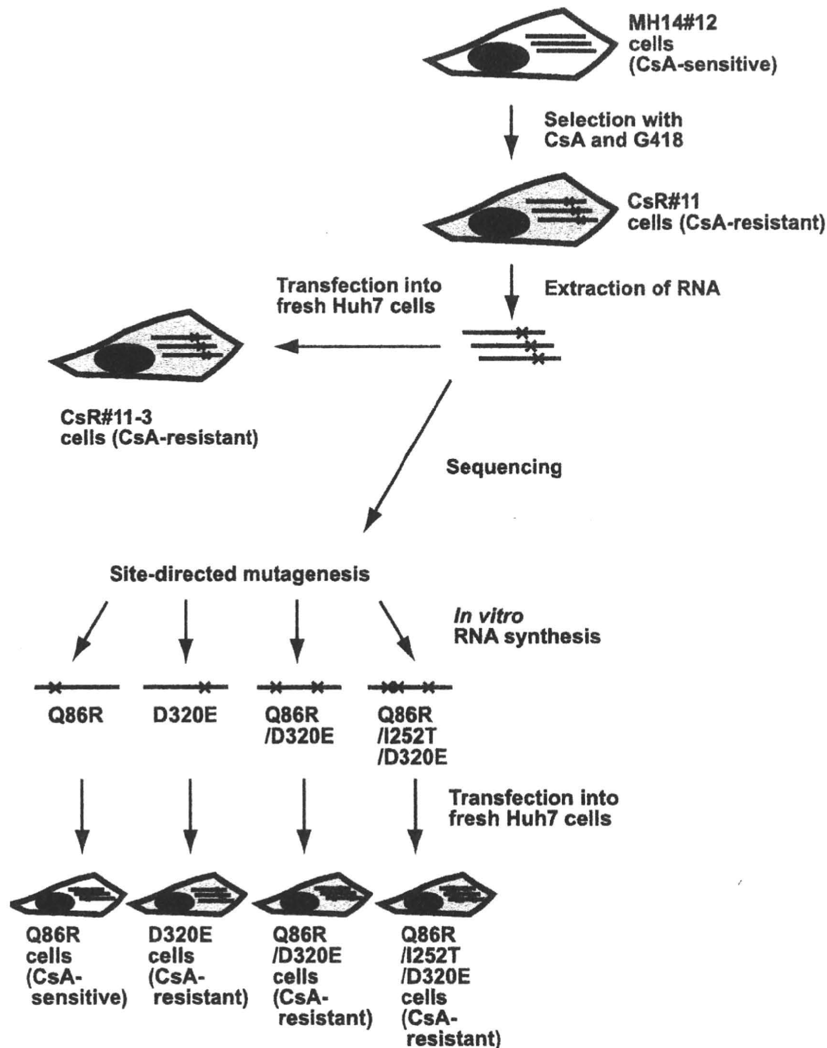

Fig. 1. Schematic diagram outlining the production of individual cell clones carrying hepatitis C virus (HCV) subgenomic replicons. MH14#12 cells, carrying wild-type HCV subgenomic repicon, were treated with 2 μg/mL cyclosporin A (CsA) in the presence of 1000 μg/mL G418 and CsR#11 cells were selected. Total RNA was extracted from CsR#11 cells and transduced into naïve Huh7 cells to select CsR#11-3 cells, and sequencing of the replicon RNA in CsR#11 cells identified mutations, Q86R in NS3, I252T in NS3, and D320E in NS5A. Site-directed mutagenesis followed by *in vitro* RNA synthesis generated HCV replicon RNA carrying Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E mutations. Transduction of the RNA into naïve Huh7 cells resulted in the production of Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E cells. The sensitivity of each replicon clone to CsA is presented as 'CsA-resistant' or 'CsA-sensitive'.

Real-time RT-PCR analysis. The 5′-non-translated region of HCV-RNA was quantified using an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA, USA), as previously described.[4]

Replicon sequencing. Total RNA from replicon cells was extracted with sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) and subjected to RT-PCR reaction using super script III (Invitrogen). The products were then amplified by dividing the whole HCV region into approximately 300 bp using appropriate primer sets, and the sequence of the entire region encoding non-structural proteins was determined.

Plasmid construction. The Q86R and I252T mutations in NS3 and the D320E mutation in NS5A were generated via site-directed mutagenesis using the following primer sets: Q86R (S) 5′-AGGACCTCGTCGGCTGGCGGGCGCC-3′ plus Q86R (AS) 5′-GGCGCCCGCCAGCCGACGAGGTCCT-3′, I252T (S) 5′-AACACCAGAACTGGGGTAAGGACCA-3′ plus I252T (AS) 5′-TGGTCCTTACCCCAGTTCTGGTGTT-3′, and D320E (S) 5′-GAGTATAATCCTCCACTGCTAGAGC-3′ plus D320E (AS) 5′-GCTCTAGCAGTGGAGGATTATACTC-3′, respectively. The PCR products carrying either Q86R in NS3, I252T in NS3, or D320E in NS5A were inserted into the NotI-MluI and MluI-XbaI sites of pMH14, respectively. The resultant plasmids were termed pMH14 (Q86R), pMH14 (I252T), and pMH14 (D320E) respectively. The double mutant carrying both Q86R and D320E mutations was produced by exchanging the MluI-XbaI region of pMH14 (Q86R) with that of pMH14 (D320E), and termed pMH14 (Q86R/D320E). The triple mutant carrying Q86R, I252T, and D320E was produced by exchanging the Not-MluI region of pMH14 (D320E) with the fragments amplified by the primer set, I252T (S) plus I252T (AS), using pMH14 (Q86R) as templates for the PCR reaction. Sequence analysis of the resultant plasmids was also undertaken for confirmation of the mutations.

***In vitro* RNA synthesis.** Wild-type and mutant RNA of pMH14 was prepared by *in vitro* transcription using the MEGAscript T7 kit (Ambion, Austin, TX, USA), as described previously.[13]

Electroporation and colony formation. 8×10^6 cells suspended in 400 μL of cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES, 2 mM EGTA, and 5 mM $MgCl_2$, together with 2 mM ATP, 5 mM reduced form of glutathione, and 1.25 % DMSO) were electroporated at 250 V, 950 μF with either 100 μg total RNA extracted from replicon cells or 5 μg RNA transcribed *in vitro* from the HCV replicon construct cDNA. Cells were then treated with 1000 μg/mL G418 for 4 weeks following electroporation.

RNAi. Validated siRNAs against CyPB were purchased from Invitrogen. SiRNA duplexes against CyPA (siCyPA161, 5′-UCUGUGAAAGCAGGAACCCUU-3′; siCyPA285, 5′-GAUG

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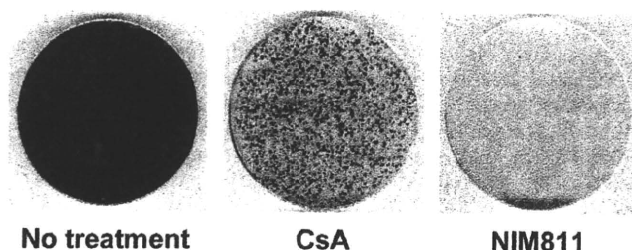

Fig. 2. Cyclophilin (CyP) inhibitor (CPI)-resistant colony emergence. MH14#12 cells were treated either without (left panel) or with 2 μg/mL CPIs, cyclosporin A (CsA) (middle panel), and NIM811 (right panel), in the presence of 700 μg/mL G418. Following 4 weeks in culture, cells were fixed and stained with crystal violet.

CCAGGACCCGUAUGCUU-3′; siCyPA459, 5′-CUUCUUG CUGGUCUUGCCAUU-3′) were synthesized (Yahima Pure Chemicals, Osaka, Japan). SiRNAs against CyP40 were purchased from Invitrogen (siCyP40-3) and from Ambion (siCyP40-4). Pre-designed siRNAs, siCyPC, siCyPE, siCyPF, and siCyPG were obtained from Ambion. Transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) with 20 nM siRNAs in the absence of CsA according to the manufacturer's protocol.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis. RT-PCR was performed as described previously[4] using the following primer sets: 5′-TGTTCTTCGACATTGCCGTC-3′ and 5′-CAGTCTTGGCAGTGCAGATG-3′ to detect mRNA for CyPA, 5′-TCTCCGAACGCAACATGAAG-3′ and 5′-CTGCGA TGATCACATCCTTC-3′to detect mRNA for CyPB, 5′-GGCGCA CTTGTGTTTTCTTC-3′ and 5′-TGCCATAGTGCTTCAGCTTG-3′ to detect mRNA for CyPC, 5′-TTTCGTGCACTGTGTACAGG-3′ and 5′-TTGGCTCTATCTGCTGTCTC-3′ to detect mRNA for CyP40, 5′-AGAGGAAGTGGACGACAAAG-3′ and 5′-GATGTCCATGTACACCTGAG-3′ to detect mRNA for CyPE, 5′-TGGAGCTGAAGGCAGATGTC-3′ and 5′-ACGTGACCG AACACAACATG-3′ to detect mRNA for CyPF, 5′-GAGTTGT CTCTTTCACAGAG-3′ and 5′-AACTGAGTATCCGTACCTCC-3′ to detect mRNA for CyPG, and 5′-ATGGGGAAGGTGAA GGTCGG-3′ and 5′-TGGAGGGATCTCGCTCCTGG-3′ to detect glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Results

Resistance emergence against individual CPIs. We have previously demonstrated the robust anti-HCV activities of CPIs, and it was reported that CPI significantly decreased HCV viral load in HCV-infected patients.[14,15] The problem of the drug-resistant HCV variants, hence, should be assessed *in vitro*, considering that practical efficacies of these inhibitors with long-term effectiveness are required in patients. In the first step of this study, we investigated the emergence of drug resistant replicon against CPIs. We treated MH14#12 cells, Huh7 cells carrying wild-type MH14 replicon with 2 μg/mL CsA, or the non-immunosuppressive analog NIM811 in the presence of 700 μg/mL G418 for 4 weeks. To visualize the appearance of drug-resistant clones, we stained cells after the selection. We observed colonies resistant to CsA, while we obtained few colonies under the treatment with the same concentration of NIM811 (Fig. 2).

Isolation and characterization of replicon cells resistant to CsA. To characterize the CsA-resistant HCV, we isolated the resistant clones following selection with 2 μg/mL CsA and 1000 μg/mL G418 for 4 weeks. We obtained several clones (named CsR cells), and examined their CsA responses. In contrast to the wild-type MH14#12 replicon cell, which showed an approximately 2-log

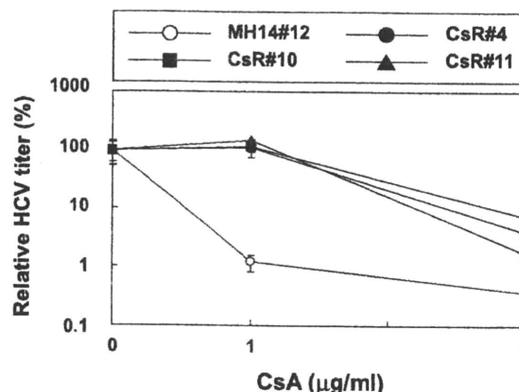

Fig. 3. Cyclosporin A (CsA) responses of the hepatitis C virus (HCV) replicon clones surviving the selection with CsA and G418. MH14#12 cells and three MH14#12-derived cell clones that survived double selection with G418 and CsA, CsR#4, CsR#10, and CsR#11, were treated with 1 and 3 μg/mL CsA for 7 days, and the HCV-RNA titers were measured by real-time RT-PCR. The data represent the percentage of HCV-RNA level in cells either untreated or treated with CsA, and the dots represent the means of three independent experiments.

reduction of HCV-RNA level by treatment with 1 μg/mL CsA for 7 days, all the clones isolated (the results of three representative clones, CsR#4, CsR#10, and CsR#11 cells are shown here) demonstrated resistant phenotypes against CsA with no significant reduction of HCV-RNA by CsA treatment at 1 μg/mL (Fig. 3). The resistance of these clones was thought to arise as a result of (1) mutations on the HCV-RNA genome or (2) alterations in cellular factors. To test the first possibility, we investigated whether HCV-RNA itself in CsR#11 could induce the CsA resistance to naïve cells. Fresh Huh7 cells were transfected with total RNA, including HCV replicon RNA, extracted from CsR#11 cells or MH14#12 cells as controls and cultured for 3 weeks in the presence of G418 (Fig. 1). The resulting colonies were isolated and propagated individually (named cell clones from total RNA of wild-type MH14#12, MH14#12-1, MH14#12-4, and MH14#12-5 cells, and those from CsR#11, CsR#11-2, CsR#11-3, and CsR#11-5 cells). The HCV-RNA titer in MH14#12-derived cells was reduced approximately to 100th by treatment with 1 μg/mL CsA for 7 days (Fig. 4). In contrast, cell clones generated from CsR#11 cells retained a normal HCV titer level after treatment with CsA, indicating that they had lost their sensitivity to CsA. Thus, it was suggested that the CsA-resistant profile in CsR#11 cells was attributed to its HCV-RNA.

D320E mutation in NS5A confers HCV replicon resistance to CsA. In order to identify the mutation in the HCV genome that resulted in the resistance to CsA, HCV subgenomic RNA isolated from CsR#11 cells was sequenced across the subgenomic region encoding non-structural proteins. We found three specific base changes that resulted in amino acid alteration including changes from glutamine to arginine, and isoleucine to threonine at positions 86 (Q86R) and 252 (I252T) in NS3, respectively, and a change from aspartic acid to glutamic acid at position 320 (D320E) in NS5A. Given that all these three mutations, Q86R, I252T, and D320E, were retained in every replicon in CsR#11-2, CsR#11-3, and CsR#11-5 cells, it is likely that they are inherited from CsR#11 cells and are associated with the acquired CsA-resistant phenotype. To examine this possibility, we synthesized replicon RNA carrying all these three mutations and established cells carrying these replicons. The resultant cell clones were named Q86R/I252T/D320E-1 and -3 cells. Treatment of these cells with 1 μg/mL CsA decreased the HCV titer only

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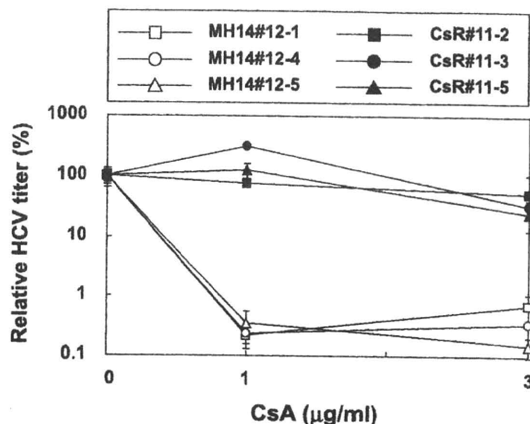
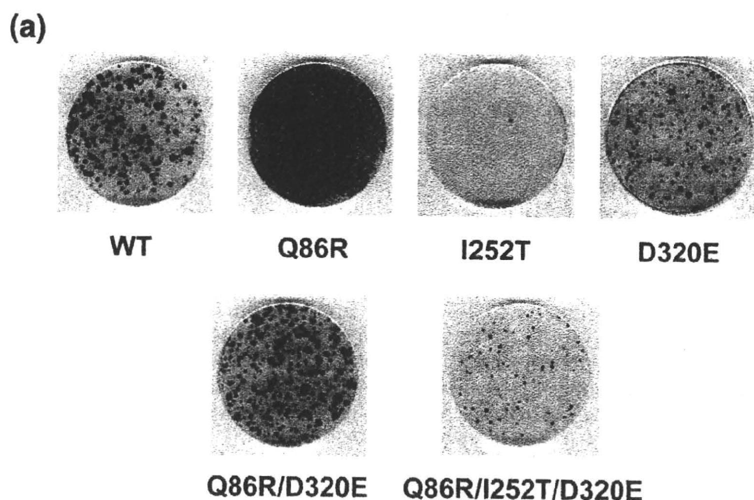


Fig. 4. Hepatitis C virus (HCV) RNA alteration contributed to cyclosporin A (CsA)-resistance. Total RNA extracted from CsA-resistant CsR#11 cells or that from wild-type MH14#12 cells as a control was transfected into Huh7 cells. Colonies established after 4-week selection with G418 were isolated, propagated individually, and tested for CsA response. Three cell clones derived from MH14#12 cells, MH14#12-1, MH14#12-4, and MH14#12-5 cells, and three cell clones from CsR#11 cells, CsR#11-2, CsR#11-3, and CsR#11-5 cells, were treated with 1 and 3 μg/mL CsA for 7 days. HCV-RNA titers were quantified by real-time RT-PCR analysis. The dots represent the means of three independent experiments.

by 1 log, in contrast to the wild-type MH14 clone, in which CsA decreased HCV-RNA by more than 2 logs under the same experimental condition (Fig. 5b). Thus, these mutations were demonstrated to confer CsA resistance; in addition to this, some cellular factors in Huh7 cells may also play minor roles in modulating the CsA sensitivities, given the result that Q86R/I252T/D320E cell clones were relatively sensitive to CsA compared with CsR#11-derived cell clones as shown in Figure 4. We next aimed to determine which of the three mutations, Q86R/I252T/D320E, was responsible for the CsA resistant phenotype, and individual mutations were engineered back into the wild-type MH14 replicon and stable replicon cells were produced as described above. Among three single amino acid mutations, the I252T mutation in NS3 resulted in a significant reduction in replication fitness (Fig. 5a), and almost failed to produce cell colonies. Cell clones harboring MH14 with both Q86R and D320E mutations, Q86R/D320E-2 and Q86R/D320E-3 cells, showed reduced sensitivity to CsA that was comparable to the levels in Q86R/I252T/D320E cells, suggesting Q86R and/or D320E mutation(s) was enough to confer the resistance. Subsequently, we treated the replicon cell clones carrying MH14 with either Q86R or D320E mutation alone, Q86R (Q86R-1 and -4 cells) and D320E (D320E-1 and -2 cells) cells, with CsA for 1 week. The titer of Q86R replicons was reduced to less than 100th by CsA treatment at a concentration of 1 μg/mL, similar to the wild type. In contrast, HCV replicon with D320E mutation in NS5A exhibited reduced sensitivity to CsA, resulting in little reduction of HCV-RNA by the treatment with 1 μg/mL CsA (Fig. 5b). Q86R mutation considerably enhanced colony



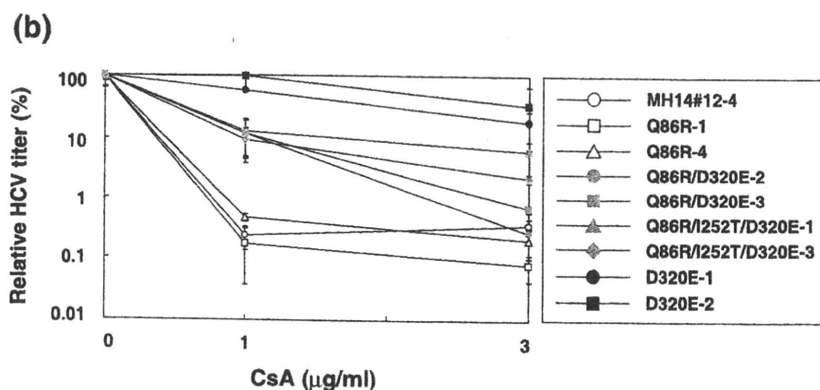


Fig. 5. The amino acid mutation D320E in NS5A conferred the cyclosporin A (CsA)-resistance to hepatitis C virus (HCV) replicons. (a) Colony formation assay for replicons carrying mutations. Five-microgram replicon RNA carrying the mutation(s), Q86R in NS3, I252T in NS3, D320E in NS5A, Q86R and D320E, or Q86R, I252T and D320E, or wild-type RNA transcribed *in vitro* were transduced into Huh7 cells. After culture with G418 for 4 weeks, colonies were stained with crystal violet. (b) Cell clones with replicons carrying indicated mutations were treated with 1 and 3 μg/mL CsA for 7 days. HCV-RNA titers were quantified by real-time RT-PCR analysis. The dots represent the means of three independent experiments. MH14#12-4, wild-type replicon; Q86R-1 and Q86R-4, replicon with Q86R mutation; D320E-1 and D320E-2, replicon with D320E mutation; Q86R/D320E-2 and Q86R/D320E-3, replicon with both Q86R and D320E mutations; Q86R/I252T/D320E-1 and Q86R/I252T/D320E-3, replicon with all three mutations, Q86R, I252T, and D320E.

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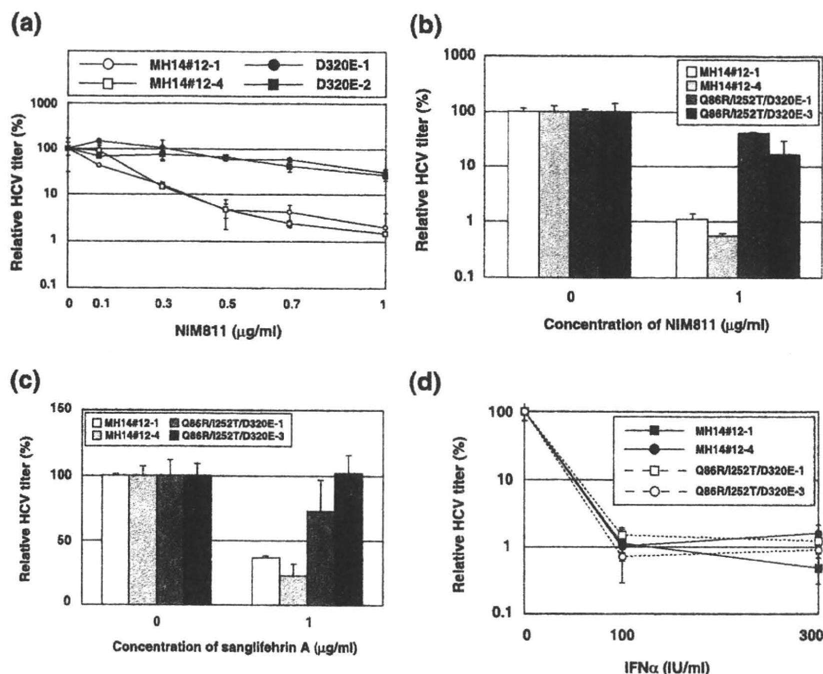

Fig. 6. Cyclosporin A (CsA)-resistant replicons demonstrated cross resistance to additional cyclophilin (CyP) inhibitors, NIM811 and sanglifehrin A (SFA), but not interferon (IFN)-α. MH14#12-1, MH14#12-4, D320E-1 and D320E-2 cells were treated with NIM811 at 0.1, 0.3, 0.5, 0.7 and 1 μg/mL (a), and MH14#12-1, MH14#12-4, Q86R/I252T/D320E-1, and Q86R/I252T/D320E-3 cells were treated with 1 μg/mL NIM811 (b), 1 μg/mL SFA (c), and 100 and 300 IU/mL IFN-α (d) for 7 days. The amounts of hepatitis C virus (HCV) RNA were quantified by real-time RT-PCR analysis. The data represent the means of three independent experiments.

formation efficiency and the D320E mutation showed little significant effect on the efficiency (Fig. 5a). Thus, the D320E mutation in NS5A was suggested as a sufficient factor to induce HCV replicon resistance to CsA, while the Q86R mutation was likely not to contribute to the resistance but to augment the efficiency of HCV replication itself.

The point mutation in NS5A conferred resistance to CPIs. Next, we examined cross-resistance between CsA and other CPIs or IFNα using the CsA-resistant replicon we produced above. Treatment with 0.1–1 μg/mL NIM811 for 7 days showed that the response to NIM811 of D320E-1 and -2 cells was less compared with that of MH14#12-1 and -4 cells, indicating that a CsA-resistant clone also acquired NIM811 resistance (Fig. 6a). A similar result was seen using Q86R/I252T/D320E cells (Fig. 6b). We then tested the anti-HCV activity of SFA, an additional CPI possessing distinct chemical backbone from those of cyclosporins.[16,17] Treatment with 1 μg/mL SFA reduced HCV replication in the wild-type cells, MH14#12-1, and -4 cells; however, it did not significantly reduce replication in Q86R/I252T/D320E cells (Fig. 6c). These results demonstrate that the CsA-resistant cells described in this study were also resistant to additional CPIs, confirming that these two compounds exerted anti-HCV effects via targeting CyP. Finally, we treated Q86R/I252T/D320E cell clones with 100 and 300 IU/mL IFNα for 7 days, and HCV-RNA titers were reduced by 2 logs in both clonal cell lines examined, Q86R/I252T/D320E-1 and Q86R/I252T/D320E-3 cells, as well as in wild-type MH14#12-derived cells, MH14#12-1, and MH14#12-4 cells (Fig. 6d). These results suggested no cross-resistance between CsA and IFNα, consistent with the previous report that the anti-HCV activity of CsA was independent of the IFNα signaling pathway.[18]

The role of CyP subtypes in HCV replication. We have previously reported that CyPB played a significant role in the efficient replication of HCV and CsA inhibited CyPB-mediated regulation of HCV replication. We have also suggested the involvement of other CyP subtypes in HCV replication.[19] To gain further insight into mechanisms underlying the anti-HCV properties of CPIs, we examined the roles of individual CyP subtypes in HCV replication in the wild-type MH14#12-1 and -4 replicon cells. In order to achieve this we knocked down CyPB with siRNAs (Fig. 7d), siCyPB-1 and -2, and found that this procedure reduced the amount of replicons to approximately half the initial level (Fig. 7c), a result consistent with the previous reports. Knockdown of CyPC, CyPE, CyPF, and CyPG (Fig. 7b) did not significantly affect the viral replication under these experimental conditions (Fig. 7a). Some groups have also suggested a role of CyPA in HCV replication.[20,21] Then, we synthesized individual siRNAs reported so far to be effective against CyPA, siCyPA-161, siCyPA-285, and siCyPA-459, and transfected them using a reagent with low cytotoxic activity to knock down endogenous CyPA (Fig. 7d). As shown in Figure 7c, the siRNAs directed against CyPA reduced HCV titers in MH14#12-1, and -4 cells. We previously observed that knockdown of CyPA little affected HCV replication in MH14 cells.[5] Here, by using a new transfection reagent with less cytotoxicity and higher knockdown efficiency, we observed the effect of CyPA knockdown on HCV replication, which suggests that CyPA-mediated regulation of HCV replication is strictly influenced by CyPA's expression level and cellular condition. Under this experimental condition, our RNAi experiments also displayed that knockdown of CyP40 (Fig. 7g), alternatively known as peptidylprolyl isomerase D (NM_005038), decreased the HCV titer (Fig. 7f) without significant cytotoxic effects, presenting CyP40 as additional cellular factor required for HCV replication.

CyPA was related to the CsA-resistant phenotype. We next asked which CyP subtype among CyPA, B, and 40 was related to the CsA resistance observed in our clones. To answer this question, we performed RNAi experiments in the CsA-resistant cell lines, CsR#11-2 and CsR#11-3 cells. Transfection of these cells with specific CyPB or CyP40 siRNAs resulted in the reduction of each subtype (Fig. 7d,g) and decreased the amount of HCV-RNA in CsR#11-derived cells and wild-type MH14#12-derived cells by approximately 50% (Fig. 7c,f). Thus, CyPB and CyP40 were likely to play roles in viral replication, even in the CsA-resistant cells. However, relative HCV titers were not reduced by CyPA knockdown in these CsA-resistant cells in contrast to the case with the wild-type replicon cells (Fig. 7c). A similar resistant phenotype to CyPA knockdown was observed in D320E

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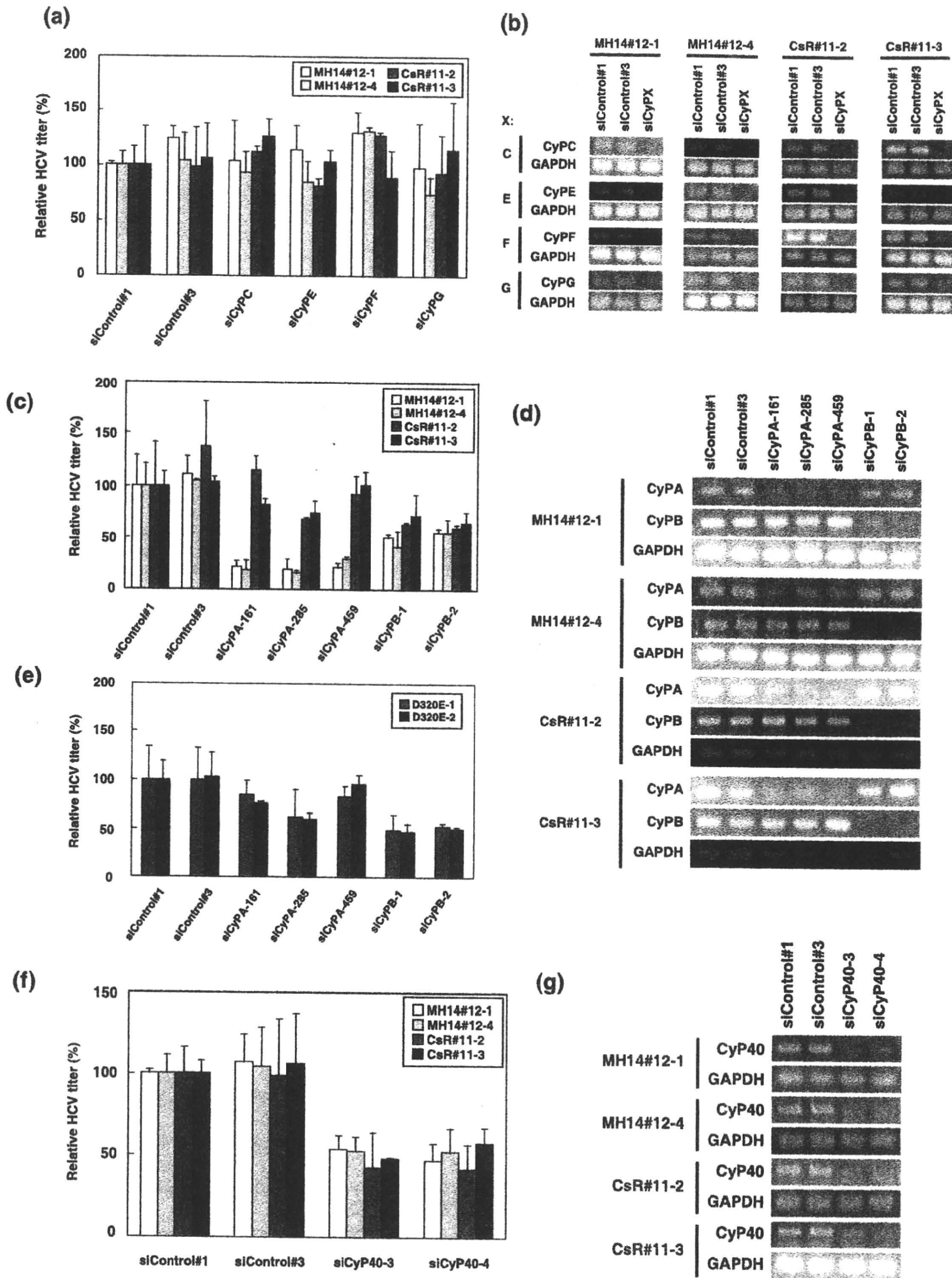

Fig. 7. Cyclophilin (CyP) subtypes related to anti-hepatitis C virus (HCV) effect of CyP inhibitor. MH14#12-derived cells, MH14#12-1 and MH14#12-4 cells, and CsR#11-2 and CsR#11-3 cells, were transfected with siRNAs specific for CyPC (siCyPC), CyPE (siCyPE), CyPF (siCyPF), and CyPG (siCyPG) (a); or those specific for CyPA (siCyPA-161, siCyPA-285, and siCyPA-459) and CyPB (siCyPB-1 and siCyPB-2) (c); or those specific for CyP40 (siCyP40-3 and siCyP40-4) (f); or randomized siRNA controls (siControl#1 and siControl#3). D320E cells were also transfected with the above siRNAs specific for either CyPA or CyPB (e). At 5 days post-transfection, the levels of HCV-RNA were quantified by real-time RT-PCR analysis. The mRNA levels of individual CyP subtypes, CyPC, CyPE, CyPF, and CyPG (CyPX corresponds to each CyP subtype indicated on the left side of the panels) (b), or CyPA and CyPB (d), or CyP40 (g) were measured using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as internal controls by RT-PCR analysis at 5 days post-transfection. The data represent the means of three independent experiments.

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cell clones (Fig. 7e), showing that CyPA was related to the CsA-resistance conferred by D320E mutation. The CsA-resistant clones obtained in this study were likely to have acquired CyPA independence for efficient HCV replication.

Discussion

Given that CPIs suppressed HCV viral load in cell culture and in patients with chronic hepatitis C,[14,15] CPIs are expected to be new anti-HCV agents. It is important to further reveal the factors related to CPI's anti-HCV activities, thinking over the practical use of CPIs with maximized efficacy and high specificity facing challenges such as side effects and the emergence of resistance to them in clinical settings. Here, we isolated and characterized a variant resistant to CPIs using a HCV subgenomic replicon system. A mutation in NS5A, D320E, was shown to confer the CPI-resistance to HCV replicon, resulting in CyPA independence for efficient viral replication. In addition, assessment of a wide range of CyP subtype knockdown experiments found CyP40 to be a new contributor to HCV replication.

Of the mutations identified, Q86R substitution in NS3 dramatically enhanced the capacity of replication. This mutation was observed as compensatory mutation[22] following the selection of replicons resistant to protease inhibitors SCH503034[23] and SCH6.[24] In addition, this mutation also appeared following the passaging of replicon cells in the absence of drug pressure.[25,26] In actuality, this mutation did not contribute to CsA resistance in the replicon cells (Fig. 5b), and thus was thought to be an adaptive mutation similar to that suggested in previous reports. I252T mutation in NS3, on the other hand, severely reduced the replicative fitness of HCV. The significance of I252T mutation under CsA treatment remains to be studied. The alteration of amino acid residue in NS5A, D320E, resulted in the conversion of HCV replicon to that of the CsA-resistant phenotype. There have been no reports of a link between NS5A and individual CyP subtype in the context of HCV replication, though mutations in NS5A were found to be keys for the acquisition of CsA resistance.[27] We have previously reported that CyPB was important for viral replication, but NS5A did not interact with CyPB in MH14 cells.[5] Indeed, in cells harboring replicons with D320E mutation, CyPB was found to contribute to viral replication but was not related to CsA resistance, as knockdown of CyPB diminished the viral titer to approximately half, similar to the case of the wild type. Therefore, other CyP molecules crucial for viral replication were suggested to be involved in the phenomenon of the CsA resistance. CyPA is another CyP subtype recently published to be critical for HCV replication in connection with viral polymerase.[20,21] Our CsA-resistant replicon cells displayed resistance to CyPA knockdown when compared to wild-type replicon, suggesting that CyPA participated in the replication process and the CsA resistance was due in part to resistance to CyPA inhibition. Therefore, it might be possible that NS5A functions coordinated with CyPA for viral replication and D320E mutation could contribute to enhancement of the relation. But NS5A was unable to bind CyPA *in vitro*.[5] NS5A might be regulated by CyPA associated with other cellular or viral factors during HCV replication. The fact that the D320 falls upon one of the two discontinuous domains needed for the interaction with NS5B to functionally modulate it[28,29] lead us to presume influence of NS5A on the reported NS5B–CyPA interaction.[21] In addition to CyPA and CyPB, which have been published to be cellular factors required for HCV replication, the results suggested that another CyP subtype, CyP40, contributed to viral replication. Acting as a molecular chaperone, it is conceivable that CyP40 directly interacts with viral proteins to boost their functions, similar to CyPA and CyPB. Heat shock protein (Hsp) 90 is a well-known chaperone forming complex with CyP40. Recently, Hsp90 was shown to be harnessed by HCV NS5A via the FK-506 binding protein 8 (FKBP8) bridge. FKBP8 is a homologous immunophilin of CyP40 that is required for viral replication.[30] This result led to the hypothesis that CyP40 serves as a linker between viral proteins and Hsp90. CyP40 is also known to associate with estrogen receptor (ESR) and we have published that ESRα escorted NS5B to replication complex (RC).[31] We also speculate CyP40 connected to ESRα may be important for the recruitment or functional reinforcement of viral and cellular factors for HCV replication in RC. Among these CyP subtypes, CyPA dependency was suggested to be one of the determinants of CsA sensitivity. Interestingly, CyPB and CyP40 play significant role in HCV replication even in CsA-resistant replicon cells. Another CPI, NIM811, is also likely to target CyPA, at least in part, to suppress HCV replication, given the cross-resistance of CsA-resistant replicon to MIN811. However, there is still also the possibility that other CyPs mediate anti-HCV effect of NIM811, which needs to be elucidated in future study.

Understanding the profile of CPI-resistance mutations in the HCV genome and the viral and cellular factors involved will aid in the progression of CPI-centered strategies preparing for the problem of drug resistance. In addition, the cells harboring CPI-resistant replicons established here may prove beneficial for further characterization of resistance mechanisms and for the screening of novel compounds with the potential of clinical application to defeat CPI-resistant variants. Also, CyP40 as a contributor to HCV replication could become another specific antiviral target. The information arising from this study is expected to contribute to the successful use of CPIs against a liver carcinogen, HCV.

Acknowledgments

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HCVと肝発癌
――HCVの感染性ウイルス粒子産生と細胞内環境
Hepatocellular carcinogenesis caused by HCV infection

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◎HCV 感染による肝癌発症には，長期にわたる慢性肝炎による継続的な細胞増殖がその原因のひとつとしてあげられる．HCV 感染細胞の癌化には，HCV 蛋白質が感染した細胞に対して何らかの影響を与えると考えられる．HCV 蛋白質のなかではコアが，現時点ではもっとも肝発癌と関連した蛋白質と考えられている．最近，培養細胞を用いた組換え体 HCV 産生実験系が確立され，著者らはこの実験系を用いて HCV のウイルス粒子産生機構について解析した．その結果，感染性 HCV 粒子の産生には細胞内の脂肪滴が重要な役割をもつことを明らかにした．このようなコアを発現している細胞では細胞内の脂肪滴の量が上昇し，中性脂肪量が上昇していることが確認された．このことは C 型肝炎肝組織中における脂肪の蓄積が感染性ウイルス粒子産生と密接に関連する可能性を示している．近年，脂肪滴の多機能性が示されているため，コアの局在化が脂肪滴の機能修飾を誘導し，細胞の機能を変化させる可能性が考えられる．

Key word：C型肝炎ウイルス，感染性ウイルス粒子産生，脂肪滴，コア蛋白質

1989 年にアメリカのベンチャー企業であるカイロン社によって，それまで非 A 非 B 型肝炎ウイルスとよばれていたウイルスの遺伝子がはじめてクローニングされ，C 型肝炎ウイルス(hepatitis C virus：HCV)と命名された[1]．これまでの疫学的研究から現在，世界保健機構(WHO)によって世界人口の 3%以上がすでにこのウイルスに感染していると報告されている．この感染者人口の多さに加えて，このウイルスに感染することによっては長期にわたる慢性肝炎が引き起こされ，それは 20～30 年後に肝硬変から肝癌へと進行する可能性が高いことから，このウイルスは人類に対する大きな脅威のひとつであると認識されている．

残念ながら HCV 感染による肝癌発症のメカニズムの詳細についてはいまだに明らかになっているわけではないが，多くの研究者の努力によりこれまでにさまざまな重要な知見が蓄積されてきている．本稿ではウイルス学的な見地から，HCV 感染による肝発癌機構について考察したいと思う．

HCV感染と肝発癌

HCV の遺伝子構造の概要が明らかとなり，このウイルスがフラビウイルス科に分類される一本鎖 RNA ウイルスであることがわかった．このウイルスの近縁種には日本脳炎ウイルスや黄熱病ウイルスなどがあり，これまでにみつかっている癌ウイルスつまり B 型肝炎ウイルスなどの DNA ウイルスや，ヒト T 細胞白血病ウイルスのようなレトロウイルスとはまったく異なるウイルスであった．

発癌ウイルスとして知られるこれらのウイルスに共通する特徴のひとつは，これらのウイルス遺伝子全体あるいはその一部が，感染した宿主細胞の染色体に組み込まれる，あるいは Epstein-Barr virus のようにエピソームとして細胞内に存在するということである．したがって，これらのウイルスによって癌化した細胞の場合，染色体上にウイルス感染の証拠としてウイルス遺伝子全体あるいは一部が検出されるか，細胞内にウイルスゲノ

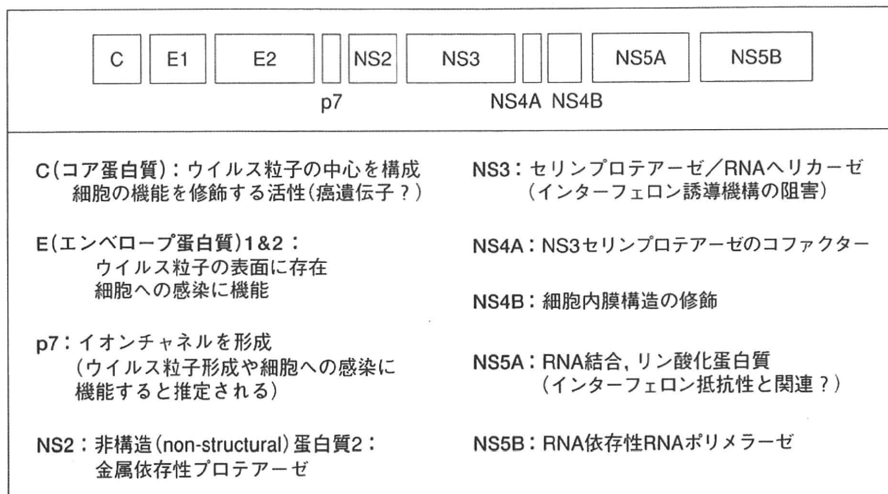

図 1　HCV蛋白質とその機能

ムが存在する．しかし，HCV を含むフラビウイルスの生活環にはその遺伝子が DNA となるステップは存在せず，実際に HCV 感染と密接に関連する肝癌組織のなかに HCV 由来 DNA はこれまでのところ見出されていない．そして肝癌組織から HCV の感染増殖を維持している細胞株を樹立することにも成功していない．また，肝癌組織のなかの HCV の RNA 遺伝子量ですら非癌部の組織よりもむしろ少ない．

このことはすくなくとも，HCV が感染した肝のなかで，癌化する細胞がどのような細胞に由来するのか正確にはわかっていないということを意味している．つまり HCV 感染と肝癌発症は密接に関連することは明らかであるにもかかわらず，癌化した細胞が実際に HCV の感染を受けたことがあるのかどうかについてさえ，なんら確証はないことになる．そこで，HCV 感染による肝癌発症には，①HCV に感染した肝細胞の癌化と，②非感染細胞の癌化，という 2 つの可能性を考える必要があるように思われる．

HCV蛋白質による細胞機能の変化

HCV 感染による肝発癌の場合，前述したようにウイルス遺伝子断片が感染細胞の染色体に挿入されることにより細胞の遺伝子発現異常が引き起こされている可能性はきわめて低い．したがって，HCV 感染細胞が癌化すると考えた場合，その遺伝子産物つまり HCV 蛋白質が感染した細胞に対して何らかの影響を与える可能性が考えられる．このことから，その細胞に対する影響を同定するために，培養細胞やマウスのような実験動物に各種 HCV 蛋白質を発現させて，その結果どのような変化が現れるのかを解析するという研究方法が用いられている．また，各 HCV 蛋白質と相互作用する細胞因子の候補分子を同定し，分子レベルからこの問題にアプローチする方法も用いられている．

図 1 に HCV 遺伝子とその産物の概略を示した．HCV は一本鎖 RNA ウイルスであり，その増殖は細胞質で行われていると考えられる．約 9,500 ヌクレオチド長の RNA ゲノムの大部分は，1 つの蛋白質読み枠(open reading frame：ORF)で占められている．ウイルス蛋白質はすべてここにコードされており，主要なウイルス蛋白質はその ORF から翻訳される前駆体ポリ蛋白質から，宿主側あるいはそのポリ蛋白質に存在するプロテアーゼ活性によってプロセッシングされて産生されてくる[2]．この ORF の 5'末端側からウイルス粒子の中心を形成するコア(C)，ウイルス粒子表面に存在するエンベロープ 1 そして 2(E1，E2)といったウイルス粒子を構成する構造蛋白質群，その C 末端側からは，p7 とよばれるイオンチャネルを形成する分子，さらにゲノム複製などに関与すると考えられる 6 種の非構造(nonstructural：NS)蛋白質

(NS2～NS5B)が産生されることがわかっている(図 1).

そのいくつかの蛋白質について培養細胞を用いたその形質転換やアポトーシスに対する影響の解析，そしてその効果を裏打ちする細胞内シグナルの修飾に関する研究が行われてきている[3]．しかし，HCV 蛋白質に関するこうした研究の結果には相反するさまざまな報告がなされ，一定の見解が得られていない場合があり，HCV 蛋白質の感染細胞に対する機能を明らかにするうえで大きな問題となっている．こうした問題は，HCV の多様性，つまり用いた HCV 蛋白質の一次構造上の多様性やそれぞれの実験で使用した細胞種あるいは HCV 蛋白質の発現方法や発現量，生理活性の検出系といった実験条件の相違に起因するのかもしれない．今後，HCV の感染増殖過程のどのような場面でこれまでに得られた結果が再現されるのか，正常な肝細胞を用いて HCV の生活環を再現する実験系を構築し，研究を進めていく必要があると思われる．

HCVコア蛋白質による形質転換

HCV 蛋白質のなかでコアが現時点でもっとも肝発癌と関連した蛋白質と考えられている．まず，コアは癌遺伝子のひとつである活性型 Ras と同時に発現されることによって，ラット胎仔線維芽細胞を形質転換するということが最初に報告された[4]．このことは形質転換実験でよく用いられるマウス BALB/c 3T3 A31-1-1 細胞を用いて著者らの研究室でも再現された[5]．さらに，コアを発現するトランスジェニックマウス(コア Tg マウス)には長期間の飼育の後に肝発癌するものが出てくることが報告された[6]．このコア Tg マウスの詳細な解析は本誌他稿に紹介されているので参照されたい．

これらの結果はすべて，コアによる細胞の形質転換能が比較的弱いものであることを示唆している．つまりコア単独では細胞の形質転換を誘導できず，コア Tg マウスでもすべての系統における早期の肝発癌が観察されないからである．それではコアは何をしているのか．このコア Tg マウス肝組織には，コントロールマウスに比較して 1.8 倍の過酸化脂質が存在することが検出されたが[7]，培養細胞においてコアを発現させた場合でも同様に過酸化脂質産生の上昇や抗酸化反応因子の遺伝子発現誘導が観察されていることから，コアの発現により遺伝子傷害性をもつ活性酸素の産生が上昇する可能性が示唆されている[8]．このどちらの場合でもミトコンドリアの損傷が認められていることから，コア発現による活性酸素の産生にはミトコンドリアの関与が示唆されている．ここでコアの細胞内局在は主として小胞体や脂肪滴であり，ミトコンドリアに局在するコアは量的に限られることから，どのようなメカニズムでコアがミトコンドリアに障害を引き起こしているのかさらに慎重に解析を進める必要があると思われる．

HCVコア蛋白質と脂肪滴

最近，培養細胞を用いた組換え体 HCV の産生実験系が確立された[9]．これは劇症肝炎患者由来の HCV 遺伝子 JFH-1(遺伝子型 2a)を試験管内で合成し，これを肝癌由来細胞 HuH-7 細胞に導入すると，その培養上清中に組換え体ウイルスが産生されるものである．この組換え体ウイルスはこの HuH-7 細胞からクローン化された HuH-7.5 細胞や HuH-7.5.1 細胞によく感染増殖し，ふたたびその上清に感染性 HCV を産生することができるのである．

著者らはこの実験系を用いて HCV のウイルス粒子産生機構について解析し，感染性 HCV 粒子の産生には細胞内の脂肪滴(「サイドメモ 1」参照)が重要な役割をもつことを明らかにした(図 2)[10]．この感染性 HCV 粒子を産生している細胞では脂

サイドメモ 1　脂肪滴

小胞体膜に由来する脂質単層膜で包まれた構造をもつ．細胞内小器官のひとつである．トリアシルグリセロールやコレステロールエステルなどの中性脂肪を含むことが知られている．これまでこれら脂肪の蓄積や供給にかかわることが知られていたが，近年，種々の細胞内小器官への脂質輸送や蛋白質の繋留や分解の場として機能することが示されてきている．

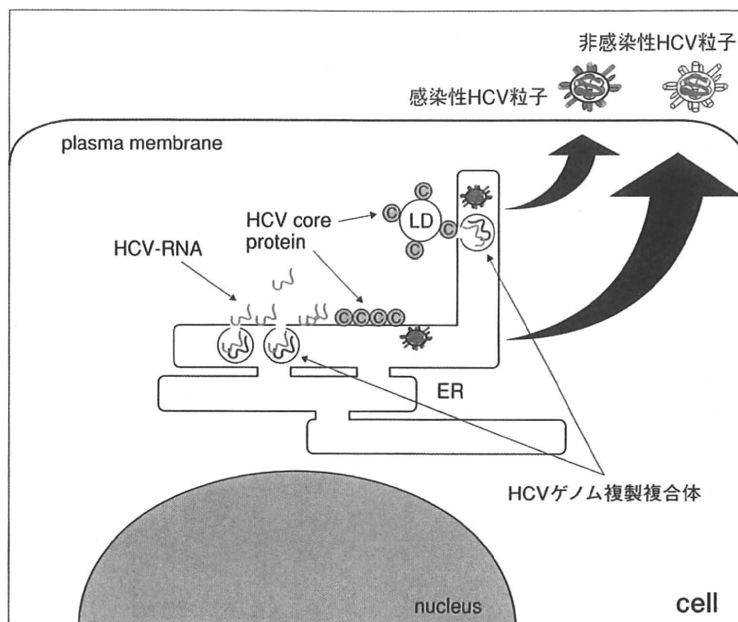

図 2　脂肪滴が関与する感染性HCV粒子産生機構の概要
ER：endoplasmic reticulum(小胞体)，LD：lipid droplet(脂肪滴).

肪滴膜上にコアが局在化しており，その周囲の膜構造にエンベロープ蛋白質や複製活性のあるHCV 遺伝子複製複合体を含む NS 蛋白質群が存在している．電子顕微鏡で観察するとウイルス粒子はその脂肪滴の周囲にある膜構造のなかに認められる．上清に含まれる HCV 粒子には浮遊密度1.12 をピークとする感染性粒子と 1.15 をピークとする非感染性粒子とが存在し，その双方にコアと遺伝子 RNA が含まれていることがわかった．しかもコアや遺伝子 RNA 量でみるかぎり非感染性粒子に比べて感染粒子の量はきわめて少ないことがわかった．組換え体 HCV 遺伝子において脂肪滴に局在化しない点突然変異をコアに導入すると，HCV 遺伝子の複製は正常であるが，他の HCV 蛋白質は脂肪滴周囲には存在せず，感染性ウイルスの産生は認められない．また，同様に NS5A にコアと相互作用しないアミノ酸変異を導入した場合には，コアは脂肪滴上に存在し HCV 遺伝子複製は認められるにもかかわらず，他の HCV 蛋白質は脂肪滴周囲には存在せず，感染性ウイルスの産生はほとんど認められない．ただし，この NS5A 変異型 JFH-1 の場合でも培養上清には HCV コアと HCV RNA を含む非感染性粒子の産生は認められるため，この系は脂肪滴とは重要な関係を有しないものであると考えられる．このような感染性ウイルス産生系においてコアを発現している細胞で可視化できる脂肪滴の数量が上昇していた(図3)．

そして感染性粒子産生細胞では，中性脂肪量が上昇していることが認められた(図 4)．このことは，上述したコア Tg マウスにおいて全例脂肪肝が認められ，コアの発現により肝に脂肪が蓄積することや慢性 C 型肝炎患者の肝に脂肪肝がよく認められることと一致すると思われる．つまり，HCV 感染肝細胞中における脂肪の蓄積は感染性ウイルス粒子産生のために必然的なものなのかもしれない．

近年，脂肪滴の機能が細胞内における中性脂肪の貯蔵であるだけでなく，細胞内膜系や種々のオルガネラへの脂質輸送や蛋白質の繋留や分解の場として機能することが示されてきている[11,12]．つまり脂肪滴にコアが局在化することがこうした脂肪滴の機能障害が活性化を引き起こし，その結果，細胞内のシグナル経路の修飾や上述したミトコンドリア損傷に関係する可能性もあるかもしれない．

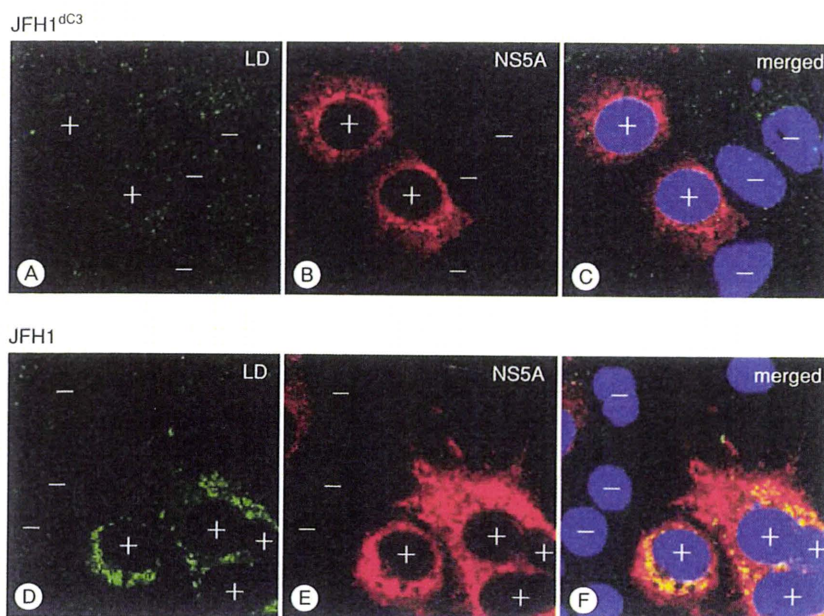

図 3　HCVコア蛋白質発現感染性HCV産生細胞における脂肪滴の誘導
コア領域を欠損したJFH1dC3遺伝子RNAあるいは野生型同等以上に感染性の高いウイルスをつくるJFH1E2FL RNAを導入したHuh-7細胞の脂肪滴の検出. RNAが導入された細胞はHCV NS5Aの発現で確認される.

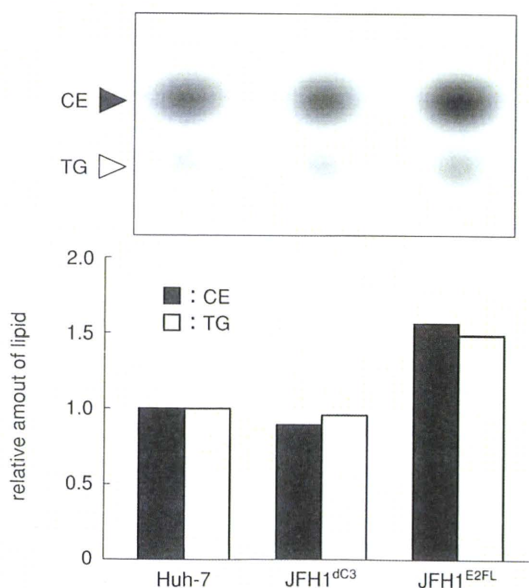

図 4　中性脂肪の蓄積[10]
未処理Huh-7細胞と図3で用いたRNAを導入した細胞内の中性脂肪を薄層クロマトグラフィーで分離定量した. CE：コレステロールエステル, TG：トリアシルグリセロール.

肝炎と肝発癌

HCV感染による肝癌の発症は20～30年という長い慢性肝炎を経過した後に起こることが知られている. ウイルス性肝炎は一般的にウイルスが感染した細胞を宿主の免疫系が攻撃し, これを破壊するという生体反応であると考えられている. 肝ではこうして排除された肝細胞は再生によって補われる. したがって, 慢性C型肝炎で長期にわたって継続的な肝細胞の破壊と再生が繰り返し行われることは, 肝細胞の増殖活性化と同じ意味をもつことになるのかもしれない.

初代培養細胞の形質転換の前段階に細胞の不死化(「サイドメモ2」参照)という継続的な細胞増殖の段階が存在するように, 継続的な細胞増殖は遺伝子変異の蓄積を引き起こす原因と考えられている. さらに, 肝細胞は恒常的に免疫系細胞群からの種々の炎症性サイトカインや遺伝子変異を引き起こす活性酸素に曝されることになると思われる. つまりHCV感染による肝発癌の原因のひとつが慢性肝炎そのものである可能性が考えられる. このことは, かならずしも癌化する細胞にHCVが感染する必要がないことを意味するかも

しれない．

しかし，慢性肝炎のなかでも自己免疫性肝炎症例の場合，肝硬変へと進行するが，肝癌の発症はウイルス性肝炎ほどではないことが知られている．自己免疫性肝炎とC型慢性肝炎の肝細胞に及ぼす影響の質的な相違は明らかではないが，すくなくともHCV感染による慢性肝炎が肝発癌への重要な要因となっており，HCVの何らかの要因がさらにその進行を加速する可能性は否定できないと考えられる．

おわりに

HCV感染による肝発癌には長期の慢性肝炎が重要な原因となっていると思われる．HCV感染が果たす役割は肝炎を誘導する以外にも，感染した細胞自体に癌化を促す作用がある可能性が考えられる．これには，HCVの感染が細胞の癌化に必要ないくつかのステップにかかわる宿主細胞の遺伝子変化を誘導することによって正常細胞を癌化させるというメカニズムが考えられている．

今回，著者らの研究から，HCVの感染性粒子産生機構そのものがヒトの肝細胞のとくに脂肪滴に変化を及ぼすことが明らかとなった．近年，脂肪滴の機能が単に中性脂肪の蓄積にとどまらず，さまざまな生命現象と関連することが示されてきている．このことから，たとえばHCVコア蛋白質の脂肪滴への局在化を抑制する方法が確立すれば，HCVの感染性粒子産生を阻害するのみならず，脂肪滴を介した細胞への影響を抑えることになるため，HCVによる肝発癌に対して効果の高い予防戦略となる可能性があると考えられる．

サイドメモ2　不死化

正常な体細胞は通常寿命をもっており，分裂を繰り返すと死ぬ場合がほとんどである．このことは細胞が分裂限界を有するということを意味するが，不死化とは細胞の分裂限界がなくなり，半永久的に分裂可能な状態になることである．癌化または形質転換とは異なり，無軌道に分裂するわけではなく，分裂には増殖因子刺激が必要であったり細胞密度が上昇しすぎると分裂を停止するなど，ある一定の秩序をもった増殖を行う．通常細胞の癌化への全段階と考えられている．

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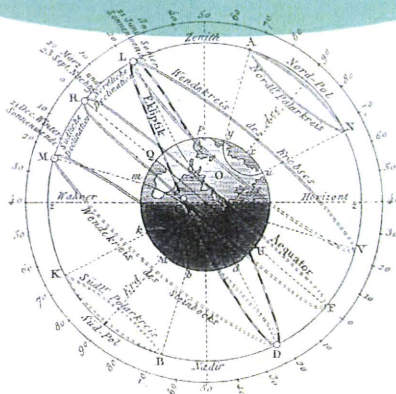
【解説】



C型肝炎ウイルスの生活環と発がん

宮成悠介[*1]，臼田信光[*2]，土方　誠[*3]，下遠野邦忠[*4, 5]

C型肝炎ウイルス (HCV) は，持続感染して慢性肝炎を発症する．慢性肝炎から肝硬変を発症，肝がんに至る場合が多い．病態の進展にはウイルスが持続的に感染していることが重要である．つまり，ウイルス複製そのものが何らかの形で病態進行に関与していると考えられる．ウイルス複製の分子機構を理解することは，病気の発症の原因を理解するために重要である．さらには持続感染を遮断して，HCV 感染を除去することによる病気の発症予防にもウイルス複製機構の解明は重要である．ここでは，HCV 複製の生活環について最近明らかにされた点を中心に概説し，最後に肝発がんとウイルス複製との関連について述べる．

C 型肝炎ウイルス (HCV) に感染すると最初急性肝炎を発症するが，そのうちの約 2 割がウイルスを排除して回復するものの，8 割の感染者ではウイルスが持続感染する結果慢性肝炎状態になる．慢性肝炎患者の約 4 分の 1 が肝硬変へと病態が進み，その半数が肝臓がんを発症する．HCV 感染により肝硬変を患う患者の約 4 分の 1 が本疾患のために命を落とすので，全体的では HCV 感染者の 15〜20% が肝疾患の犠牲になると推定される．

HCV Life Cycle and Its Pathogenesis
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C 型慢性肝炎の治療にインターフェロンとリバビリン（核酸類似体）の併用療法が功を奏している．治療を完了した約半数の患者からウイルスが消えており，それらのヒトではその後の肝障害も見られない．一般に，ヒトにがんをひき起こすウイルスは排除するのが困難であることを考えると，半数近くの患者から HCV を排除できるというこれまでの成績は，今後抗 HCV 剤の開発が進めばさらに効果的にウイルスを排除できる可能性を示唆する．また，ウイルス複製の持続性が疾患の維持および悪性化に関わるので，複製そのものが細胞の増殖に影響を及ぼしており，そのことが疾患と関連すると考えられる．したがって，HCV 複製の分子機構およびその過程に宿主がどのように関係してくるのかを理解することは，病気発症の分子機構解明の一助になると期待できる．そのような期待を抱いて，以下に HCV の複製の分子機構を中心に概説し，最後に疾患との関連を推測したい．

HCV ゲノムとタンパク質

HCV はフラビウイルス科に属する．本科のウイルスはプラス鎖からなる RNA をゲノムにもち，ウイルス粒子はエンベロープに覆われている．ゲノム内にある最も大きいタンパク質の読み枠からすべてのウイルスタンパ

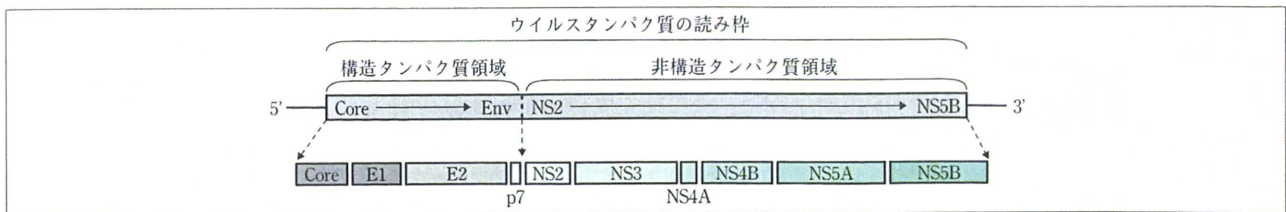


図1■HCVゲノムの構造（上）と産生されるタンパク質（下）
ゲノムは約10,000ヌクレオチドからなるプラス鎖RNAである．ゲノム全体の9割を占めるタンパク質読み枠のN端から前駆体ウイルスタンパク質が翻訳され，それが切断されて個々のタンパク質になる．Core：コア，Env：外膜タンパク質，NS：非構造タンパク質（non-structural proteinsの略）

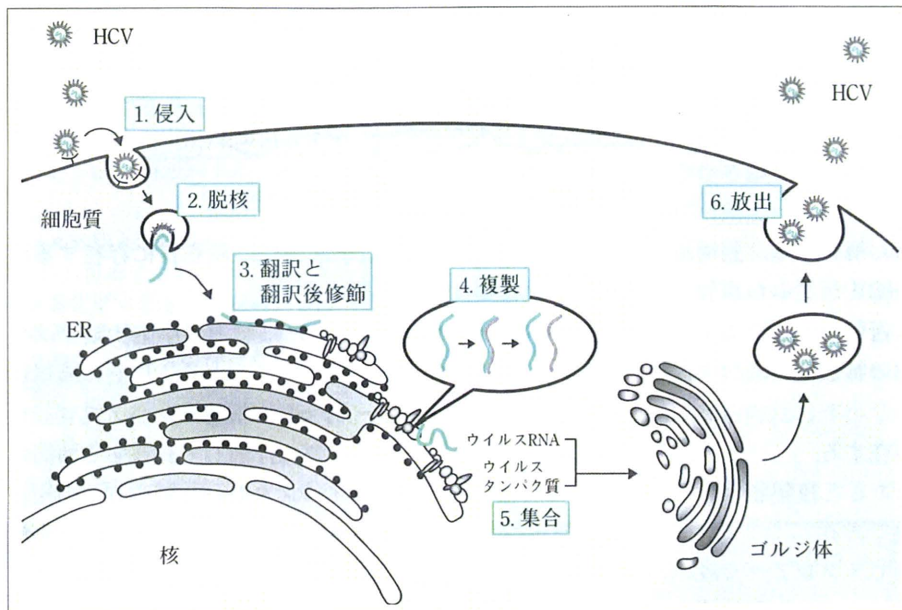


図2■HCVの複製サイクル
HCVは細胞表面の受容体を介して細胞に取り込まれると考えられる．それらの細胞タンパク質のうち，侵入の後期には，Claudin1やCD81が関与すると考えられる．細胞内に入ったウイルス核様体内のゲノムRNAがmRNAとして働き，図1に示したタンパク質が産生される．ウイルスゲノム複製は本文で述べるように，小胞体膜内の複製複合体内で行なわれる．ER：小胞体

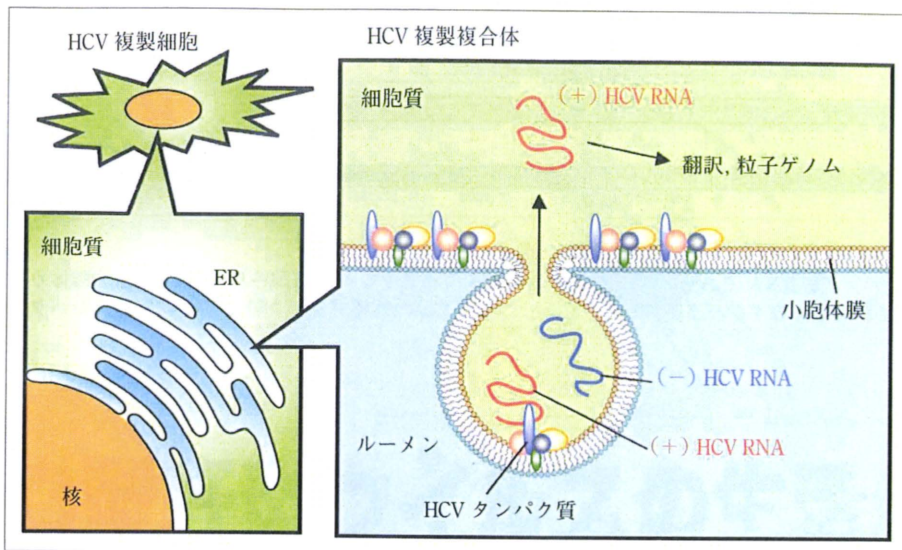
ク質が産生される．ウイルスタンパク質は，ウイルス自身のペプチダーゼによる切断あるいは宿主のシグナルペプチダーゼによる切断，糖鎖付加などの翻訳後修飾を経て最終的に機能する成熟タンパク質になる（図1）．

HCVは感染細胞内の細胞質で複製する（図2）．感染に際しては細胞表面に存在する受容体を介すると考えられる．これまでのところ，複数の候補遺伝子が報告されているが，それらが細胞に接触し感染が成立する過程でどのように機能しているかについては，十分に理解されているとはいいがたい．しかし，それらの中で，CD81, Claudin 1などは，ウイルス侵入後期に関わる重要な細胞側因子であると考えられる[1]．

ウイルスタンパク質としては約10種類産生されるが，それらを2つのグループに分けることができる．1つは粒子構成タンパク質（構造タンパク質）であり，もう1つは感染細胞内でウイルス複製に関与するタンパク質（非構造タンパク質）である．構造タンパク質は，コア，外被膜タンパク質（E1, E2）などがある（もう1つのタンパク質，p7についてもウイルス粒子構成タンパク質であると示唆されるが，まだ決定的ではない）．非構造タンパク質は約5～6種類からなり，それぞれのタンパク質は独立した機能をもつが，非構造タンパク質全体としての最も重要な機能は，それらが複合体を形成してウイルスゲノムの複製およびmRNAを合成する場（複製複合体）を構築することである．

ウイルスゲノム複製は小胞体周辺に感染して新たに構築される特殊な構造体の中で行なわれる[2～4]

ウイルス粒子産生にまでは至らないがゲノム自身は細胞内で自立的に複製できる欠失ウイルスゲノムの存在が知られている[5]．そのゲノムを導入した細胞においては常時ウイルスゲノムの複製が見られ，細胞が分裂すると同時にウイルスゲノムも娘細胞に分配され，そこで複製を繰り返すようになる．このような細胞の切片を電子顕微鏡で観察すると，小胞体膜の一部の膜構造が著しく変形した像が見られる[6]．正常細胞にはこのような像は観



HCV 複製細胞（左図）の小胞体膜部分を拡大（右図）し，その中に存在するHCV 複製複合体のイメージを記載した．小胞体ルーメン内に突き出した形の膜構造体（図で円形の形で示した）内でウイルスの核酸合成が行なわれる．核酸合成に関与するウイルスタンパク質は細胞内の全ウイルスタンパク質のうちのわずかの量である．ほかのウイルスタンパク質は細胞質側に露出していると考えられる．合成されたウイルスRNA は細胞質に放出され，mRNA やゲノム RNA として機能する．

図3■HCV 複製複合体の模式図

察されない．生化学的な手法を用いた解析から，膜構造が変化したこの本体は，その中でHCV ゲノムが複製する活性をもった膜様構造体（ゲノム複製複合体）であると考えられる[4]．ゲノム複製複合体の特徴として次のようなことがあげられる．

①細胞質内の不溶性画分として存在する．

②NP40 などの界面活性剤で処理すると複製能が失われるので，膜様構造が機能的に重要である．

③細胞膜を部分的に破壊したあとでヌクレアーゼ液の入った緩衝液で処理すると，リボソーム RNA が破壊される条件下でも HCV RNA は壊れない．しかし，このとき，界面活性剤を共存させると速やかに壊れるので，HCV RNA は膜成分に保護された状態として存在している．

④細胞質に外来的にプロテアーゼを導入し，細胞質内の多くのタンパク質が加水分解を受ける条件下で処理しても，わずかのウイルスタンパク質は分解されずに残る．しかし，界面活性剤共存下ではすべてのウイルスタンパク質が消える．つまり，一部のタンパク質は膜成分に覆われた中に存在していると考えられる．

⑤界面活性剤を加えないで，大部分のウイルスタンパク質がプロテアーゼで加水分解された環境下でも，ウイルス RNA の合成には影響を受けない．

これらのことから，HCV RNA 合成は小胞体膜構造に覆われてつくられた複製複合体の中で行なわれると考えられる[4]（図3）．ウイルスタンパク質の中で，NS4B が小胞体膜に作用して膜の構造を変化させることが知られている[7]．

HCV のウイルス粒子産生には細胞質に存在する油滴が重要な役割を果たす[8]

HCV タンパク質の多くは，細胞内の小胞体膜タンパク質マーカーと局在が一致することから，小胞体膜周辺に局在するといえる．一方，HCV 構造タンパク質コアを単独に発現させると，細胞質に浮遊して存在する油滴の周りに局在する[9,10]．ウイルスタンパク質の細胞内局在とウイルス増殖との関連については不明である．感染性ウイルスを産生させる培養細胞系で個々のウイルスタンパク質の局在を調べ，その局在を人為的に変化させたときにウイルス産生にどのような影響を与えるかを調べることで，ウイルスタンパク質の細胞内局在がウイルス複製能に与える影響を調べることが可能である．

2年ほど前に樹立された感染性 HCV ゲノム（JFH1 と呼ばれている）RNA を培養細胞（HuH7：ヒト肝臓がん由来細胞株）に導入すると，ウイルス粒子が産生される[11]．これまで，培養細胞を用いた HCV 感染・増殖系がなかったので，この系の樹立はウイルスの増殖を解析するうえで大変重要である．HCV ゲノム RNA を導入した細胞内でのコアタンパク質の細胞内局在を調べると，これまでの報告通りに，油滴の周りに局在が見られる．また，非構造タンパク質 (NS3-NS5B) の多くは小胞体に局在している．しかし，詳細に調べると，小胞体近辺の油滴の周りにも NS タンパク質の局在が観察される[8]．この細胞培養液からウイルス粒子を回収して，ショ糖密度勾配遠心により浮遊密度を調べると，密度 1.15 (g/ml) の画分に大部分のウイルスが検出される．しかし，この画分のウイルスには感染性がない．一方，密度 1.12 (g/ml) の画分には，わずかな粒子しか検出され