厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業

Ｃ型肝炎ウイルスの非構造蛋白５Ａを標的とした新規治療法の開発に関する研究

平成２２年度　総括研究報告書

研究代表者　　政木　隆博

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Ⅰ．総括研究報告

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）

総括研究報告書

C型肝炎ウイルスの非構造蛋白５Ａを標的とした新規治療法の開発に関する研究

研究代表者　　政木　隆博　　国立感染症研究所　ウイルス第二部　主任研究官

研究要旨：本研究では、HCVのリン酸化蛋白NS5Aを標的とした新規治療法の開発を目的とし、以下の4項目、(1)NS5A蛋白と強く相互作用するプロテインキナーゼの網羅的探索、(2)NS5A蛋白をリン酸化するプロテインキナーゼの同定、(3)同定されたプロテインキナーゼがHCVゲノム複製、粒子形成に与える影響の解析、(4)HCVゲノム複製を制御するNS5A蛋白結合ペプチドの同定、を行った。その結果、NS5A蛋白と相互作用し、NS5A蛋白をリン酸化する新規セリン/スレオニンプロテインキナーゼを同定し得た。さらに、この中から感染性HCV産生を制御する2種類の新規プロテインキナーゼ(CK1α、CK1ε)を見出した。また、HCVゲノム複製を中等度に抑制する9種類のNS5A蛋白結合ペプチドを取得した。

本研究は、HCVゲノム複製、粒子形成機構の解明に加え、新たな創薬標的の同定や創薬開発に道を拓く可能性を有する。

A. 研究目的

C型肝炎ウイルス(HCV)感染者は現在本邦で約200万人にのぼると推定される。感染後は持続感染により肝炎が慢性化し、肝硬変を経て高率に肝細胞癌を合併することが知られており、公衆衛生上きわめて重要な病原ウイルスである。肝発癌を防ぐためにはウイルス排除が必須である。現在の主な治療法はペグインターフェロンとリバビリンによる併用療法であるが、その効果は未だ充分とはいえず、従来の抗HCV薬と異なる作用点をもつ新規治療法の開発は厚生労働行政上急務である。

HCVの非構造(NS)蛋白であるNS5A蛋白は、HCVの複製増殖やインターフェロン感受性、病原性発現などに関与する多機能蛋白である。リン酸化蛋白であり、NS5A蛋白のリン酸化はウイルスゲノム複製や感染性ウイルス粒子の形成に重要な役割を担うことが報告されている。しかしながら、酵素としての機能が明確なNS3、NS5B蛋白に比べ、NS5A蛋白を標的とした阻害薬の開発は遅れている。そこで、本研究では、NS5A蛋白を標的とした新規治療法の開発を目的とする。具体的には、(1)NS5A蛋白のリン酸化に着目し、リン酸化に関与する責任プロテインキナーゼの同定と創薬標的としての妥当性の検証、(2)HCVゲノム複製、粒子形成を制御するNS5A蛋白結合ペプチドの開発・取得、並びに、創薬への応用可能性の検証、を行った。

B. 研究方法

NS5A蛋白のリン酸化に関与するプロテインキナーゼの同定に関しては、まず、(1)NS5A蛋白と強く相互作用するプロテインキナーゼの探索を行った。次に、(2)強い相互作用が認められたプロテインキナーゼに関して、NS5A蛋白に対するリン酸化能を調べた。最後に、(3)同定されたプロテインキナーゼがHCVゲノム複製、粒子形成に与える影響を培養細胞を用いて解析した。

HCVゲノム複製、粒子形成を制御するNS5A蛋白結合ペプチドの取得に関しては、(4)東京大学先端科学技術研究センターとの共同研究で既に取得しているNS5A蛋白結合ペプチドをHCVサブゲノミックレプリコン細胞に導入し、HCVゲノム複製に及ぼす影響を解析した。

具体的な実験・解析方法は以下の通りである。

(1) <u>NS5A蛋白と強く相互作用するプロテインキナーゼの探索</u>

JFH-1株(遺伝子型2a)のNS5A蛋白をコムギ胚芽無

細胞転写・翻訳系で合成し精製した。また、404種類のヒトプロテインキナーゼを包括するcDNAライブラリーから同様の方法で精製プロテインキナーゼを取得した。精製NS5A蛋白と精製プロテインキナーゼの相互作用はハイスループットな定量解析が可能である*AlphaScreen*法を用いて解析した。

(2) NS5A蛋白に対するリン酸化能の評価

NS5A蛋白に対するリン酸化能の評価は、精製プロテインキナーゼを[γ-^{32}P]ATP存在化において精製NS5A蛋白と混和し、SDS-PAGEで展開後、オートラジオグラフィーを用いてリン酸化NS5A蛋白のバンドを検出することにより行った(*in vitro*リン酸化アッセイ)。

(3) HCVゲノム複製、粒子形成に与える影響の解析

HCVゲノム複製能はサブゲノミックレプリコンRNAを用いて、また、粒子形成能は全長HCV RNAもしくは感染性ウイルス粒子を用いて解析した。細胞にはヒト肝癌由来細胞株(HuH-7)及びその派生株を使用した。HCV RNA導入細胞、HCV感染細胞におけるプロテインキナーゼの発現をsiRNAによりノックダウンし、HCVゲノム複製能、粒子形成能に与える影響を解析した。また、NS5A蛋白のリン酸化状態をウエスタンブロッティング法により解析した。培養上清中の感染性ウイルス粒子量の測定は、培養上清を非感染細胞に処理後、感染巣(フォーカス)をカウントし、1 mLあたりのフォーカス形成単位(FFU)を算出することにより行った。

(4) HCVゲノム複製を制御するNS5A蛋白結合ペプチドの取得

大腸菌発現システムを利用し、JFH-1株NS5A蛋白のdomain I 領域を発現させ、精製した。この精製NS5A蛋白を用いて東京大学先端科学技術研究センターと共同で11種類のNS5A蛋白結合ペプチドを取得した。次に、取得ペプチドをProtein Transduction Kit (Jena Bioscience)を用いてHCVサブゲノミックレプリコン細胞に導入し、各NS5A蛋白結合ペプチドの抗HCV作用(今年度はHCVゲノム複製に対する作用)を解析した。

(倫理面への配慮)

各種研究材料の取り扱い及び組換えDNA実験は国立感染症研究所内のバイオリスク管理委員会、組換えDNA実験委員会等の承認を受けて行った。組換えHCVの作製は遺伝子組換え生物等の第二種使用等にあたるため「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律」(平成15年法律第97号)の規定に従って申請を行い、承認を得た(大臣確認通知番号 大20-9 平成18年1月23日付17国文科振第47号、及び平成18年8月10日付18国文科振第16号)。

C. 研究結果

(1) NS5A蛋白と強く相互作用するプロテインキナーゼの探索

*AlphaScreen*法による解析において、発光シグナル強度1,300以上を強固な蛋白間相互作用と想定した時、このカットオフ値以上のシグナル強度を示したプロテインキナーゼは89種類であった。このうち79種類がNS5A蛋白のリン酸化に重要とされるセリン/スレオニンプロテインキナーゼであり、このグループの中にはNS5A蛋白をリン酸化すると既に報告されているカゼインキナーゼ(CK)2の触媒サブユニットであるCK2α2も含まれていた。

(2) NS5A蛋白に対するリン酸化能の評価

*AlphaScreen*解析でスクリーニングされた79種類のセリン/スレオニンプロテインキナーゼに対して*in vitro*リン酸化アッセイを行ったところ、9種類にNS5A蛋白に対する強いリン酸化活性が認められた。

(3) HCVゲノム複製、粒子形成に与える影響の解析

NS5A蛋白をリン酸化する9種類のプロテインキナーゼがHCV生活環に役割を有するか否かを調べるために、各プロテインキナーゼの細胞内発現をノックダウンした状態でHCVを感染させ、感染後のウイルス粒子産生量を解析した。ヒト肝癌由来細胞株において発現が認められなかったり、ノックダウンにより著しい細胞障害を示したプロテインキナーゼはこの解析から除外し、7種類のプロテインキナーゼを対象とした。CK2α2ノックダウン細胞から分泌される感染性ウイルス粒子量(ウイルス感染力価)は、mock処理細胞もしくはコントロールsiRNA導入細胞の約1/2に抑制された。この結果はCK2が感染性HCV粒子の産生を制御するという過去の報告内容を支持するものであった。さらに、ノックダウンによりCK2α2以上に感染性ウイルス粒子分泌量を低下させる新規プロテインキナーゼを2種類見出した(CK1α及びCK1ε)。次に、これらのプロテインキナーゼがHCV生活環の中のどのステップに関わっているのかをより詳細に調べるために、プロテインキナーゼノ

ックダウン細胞におけるRNA複製能をサブゲノミックレプリコンシステムを用いて、また、HCV粒子形成能をウイルス感染が成立しないHuH-7細胞(Huh7-25細胞)を用いたHCV産生システムで解析した。HCV RNA複製はレポーターとしてレプリコンに挿入されたルシフェラーゼ遺伝子の発現を指標にして定量的に評価した。2種類の新規プロテインキナーゼ及びCK2α2ノックダウン細胞におけるRNA複製能はmock処理細胞もしくはコントロールsiRNA導入細胞の複製能と同程度であり、これらのプロテインキナーゼの作用点はゲノム複製のステップではないことが示唆された。HCV粒子形成能の解析では、全長HCV RNAをプロテインキナーゼsiRNAとともにエレクトロポレーション法で細胞に導入し、導入後3日目の上清中コア蛋白量を測定した。2種類の新規プロテインキナーゼ及びCK2α2ノックダウン細胞から分泌されるコア蛋白量はmock処理細胞もしくはコントロールsiRNA導入細胞における分泌コア蛋白量の1/3～1/2に減少し、ウイルス粒子形成過程がこれらのプロテインキナーゼの作用点である可能性が示唆された。最後に、2種類の新規プロテインキナーゼが培養細胞内においてもNS5A蛋白のリン酸化に関与するか否かを調べるために、プロテインキナーゼノックダウン細胞にHCVを感染させ、NS5A蛋白のリン酸化状態を解析した。CK1αノックダウン細胞では、コントロールsiRNA導入細胞と比べて、高リン酸化型NS5A蛋白の発現低下及び高リン酸化型NS5A蛋白/低リン酸化型NS5A蛋白比の減少を認めた。一方、CK1εノックダウン細胞におけるNS5A蛋白のバンドパターンはコントロールsiRNA導入細胞のNS5A像と同様のパターンを呈していた。

(4) HCVゲノム複製を制御するNS5A蛋白結合ペプチドの取得

取得した11種類のNS5A蛋白結合ペプチドのうち、9種類においてHCVゲノム複製の有意な抑制効果を認めたが、その抑制効果はコントロール群と比べて何れも30-50%程度であった。顕著な細胞障害を示したペプチドはなかった。

D. 考察

(1) リン酸化に関与する責任プロテインキナーゼの同定

NS5A蛋白はHCVの複製増殖やインターフェロン感受性、病原性発現などに関与する多機能蛋白である。リン酸化蛋白であり、NS5A蛋白のリン酸化はウイルスゲノム複製や感染性ウイルス粒子の形成に重要な役割を担うことが報告されている。したがって、NS5A蛋白のリン酸化を制御するプロテインキナーゼの同定は、HCV生活環をより理解する上で重要であることに加え、新たな創薬ターゲットとしても魅力的である。

阻害剤や酵母由来の精製プロテインキナーゼを用いた解析から、現在までにAKT、p70S6K、MEK、CK1、CK2など数種類のプロテインキナーゼがNS5A蛋白のリン酸化に関与するものとして報告されている。しかし、いずれの報告も解析対象のプロテインキナーゼ数は数十～百前後であり、また、HCV生活環への関与についても十分な検討がなされていない。そこで、今回われわれは404種類のヒトプロテインキナーゼを対象とし、NS5A蛋白に対するリン酸化能を有し、かつ、HCV生活環に関与するプロテインキナーゼの同定を試みた。同定された3種類のプロテインキナーゼの中にはCK2の触媒サブユニットであるCK2α2が含まれていたが、CK2はNS5A蛋白のリン酸化とHCV粒子形成に関与することが報告されており、本解析結果の妥当性が高いことを示している。

今回の解析で同定された3種類のプロテインキナーゼ(CK1α、CK1ε、CK2α2)は、いずれもsiRNAによるノックダウンで複製活性には影響を与えずにウイルス粒子分泌量を低下させたことから、HCV生活環の後期過程であるウイルス粒子形成（もしくはそれ以降のステップ）に関与し、この過程を正に制御している可能性が示唆された。さらに、CK1αのノックダウンはNS5A蛋白の高リン酸化を著しく抑制しており、このプロテインキナーゼの粒子形成過程への作用はNS5A蛋白の高リン酸化制御を介している可能性が考えられた。最近、台湾の研究チームがサブゲノミックレプリコン細胞を用いて1,210種類のヒトプロテインキナーゼ及びホスファターゼを対象とした網羅的RNAiスクリーニングを行い、HCVゲノム複製にPolo-like kinase 1 (Plk1)というプロテインキナーゼが関与することを報告した。Plk1の作用点はHCVの複製過程であるが、その作用はわれわれが同定したCK1αと同様NS5A蛋白の高リン酸化制御を介する。NS5A蛋白の高リン酸化にはその中央領域に存在する複数のセリン残基が関与すると報告されているが、同じリン酸化パターンでも責任プロテインキナーゼやNS5A蛋白のリン酸化部位の違いにより

HCV生活環における作用点が異なる可能性は充分に考えられる。この相違を明らかにするためには、今後、責任プロテインキナーゼによるリン酸化部位の同定や同定部位のリン酸化がHCV生活環に与える影響につき解析する必要があるであろう。

NS5A蛋白がHCV粒子形成に関与する過程においてキャプシド蛋白であるコア蛋白との相互作用や粒子形成の場である脂肪滴周辺膜への局在は必須であり、いずれにもNS5A蛋白のリン酸化が重要であると考えられている。同定されたプロテインキナーゼがNS5A蛋白のリン酸化制御を介して粒子形成過程のどのステップに関与しているのかを現在解析中である。また、HCV侵入過程に関与するプロテインキナーゼの報告もあることから、同定プロテインキナーゼがこの侵入過程にも役割を有するかどうか解析予定である。

(2) HCVゲノム複製、粒子形成を制御するNS5A蛋白結合ペプチドの取得

取得した11種類のNS5A蛋白結合ペプチドのうち、9種類においてHCVゲノム複製の有意な抑制効果を認めたが、強力な抑制効果は認めず、何れも30-50%程度の抑制率であった。今後は、複数のペプチド導入による相加・相乗効果の検討、取得ペプチドがHCV粒子産生に及ぼす影響について解析を加える予定である。

E. 結論

NS5A蛋白と相互作用し、NS5A蛋白をリン酸化する新規セリン/スレオニンプロテインキナーゼを網羅的手法により同定し、この中から感染性HCV産生を制御する2種類の新規プロテインキナーゼ(CK1α、CK1ε)を見出した。また、HCVゲノム複製を中等度に抑制する9種類のNS5A蛋白結合ペプチドを取得した。

本研究は、HCVゲノム複製、粒子形成機構の解明に加え、新たな創薬標的の同定や創薬開発に道を拓く可能性を有する。

F. 健康危険情報

特記事項なし。

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H. 知的所有権の出願・取得状況

1. 特許取得

なし。

2. 実用新案登録

なし。

3. その他

なし。

Ⅱ．研究成果の刊行に関する一覧表

雑誌

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Masaki T, Suzuki R, Saeed M, Mori K, Matsuda M, Aizaki H, Ishii K, Maki N, Miyamura T, Matsuura Y, Wakita T, Suzuki T	Production of infectious hepatitis C virus by using RNA polymerase I-mediated transcription	Journal of Virology	84	5824-5835	2010
政木隆博、松永智子、高橋宏隆、加藤孝宣、遠藤弥重太、脇田隆字、澤崎達也、鈴木哲朗	HCV NS5A蛋白質のリン酸化に関与する新規セリン/スレオニンプロテインキナーゼの探索	消化器内科	51	627-631	2010
鈴木哲朗、原弘道、相崎英樹、鈴木亮介、政木隆博	C型肝炎ウイルスの複製と粒子形成	ウイルス	60	87-92	2010

Ⅲ. 研究成果の刊行物・別刷

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Chaperonin TRiC/CCT participates in replication of hepatitis C virus genome via interaction with the viral NS5B protein

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ABSTRACT

To identify the host factors implicated in the regulation of hepatitis C virus (HCV) genome replication, we performed comparative proteome analyses of HCV replication complex (RC)-rich membrane fractions prepared from cells harboring genome-length bicistronic HCV RNA at the exponential and stationary growth phases. We found that the eukaryotic chaperonin T-complex polypeptide 1 (TCP1)-ring complex/chaperonin-containing TCP1 (TRiC/CCT) plays a role in the replication possibly through an interaction between subunit CCT5 and the viral RNA polymerase NS5B. siRNA-mediated knockdown of CCT5 suppressed RNA replication and production of the infectious virus. Gain-of-function activity was shown following co-transfection with whole eight TRiC/CCT subunits. HCV RNA synthesis was inhibited by an anti-CCT5 antibody in a cell-free assay. These suggest that recruitment of the chaperonin by the viral nonstructural proteins to the RC, which potentially facilitate folding of the RC component(s) into the mature active form, may be important for efficient replication of the HCV genome.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases, such as chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma (Hoofnagle, 2002; Manns et al., 2006; Saito et al., 1990; Seeff and Hoofnagle, 2003). HCV is an enveloped positive-strand RNA virus belonging to the *Hepacivirus* genus of the Flaviviridae family. Its genome of ~9.6 kb encodes a polyprotein precursor of ~3000 amino acids (aa) (Suzuki et al., 2007; Taguwa et al., 2008). The precursor polyprotein is post- or cotranslationally processed by both viral and host proteases into at least ten viral products. The nonstructural (NS) proteins NS3–NS5B are necessary and sufficient for autonomous HCV RNA replication. They form a membrane-associated replication complex (RC), in which NS5B is the RNA-dependent RNA polymerase (RdRp) that is responsible for copying the RNA genome of the virus during replication. The HCV RC has been detected in detergent-resistant membrane (DRM) structures, possibly in a lipid-raft structure (Aizaki et al., 2004; Shi et al., 2003). Cell-free RC replication activity has also been demonstrated in crude membrane fractions of HCV subgenomic replicon cells (Aizaki et al., 2004; Ali et al., 2002; Hara et al., 2009; Hardy et al., 2003; Yang et al., 2004); these cell-free systems provide semi-intact RdRp assays for biochemical dissection of viral replication.

In general, any process that occurs during viral replication is dependent on the host cell machinery and requires close interaction between viral and cellular proteins. Although evidence that host cell factors interact with HCV NS proteins and are involved in viral replication is accumulating (Moriishi and Matsuura, 2007), the cellular components of HCV RC and their functional roles in viral replication are not fully understood.

Recently, using comparative proteome analysis, we identified 27 cellular proteins that were highly enriched in the DRM fraction of HCV replicon cells relative to parental cells. Subsequent analyses demonstrated that one of the identified proteins, creatine kinase B, a key ATP-generating enzyme, is important for efficient replication of the HCV genome and for production of the infectious virus (Hara et al., 2009).

In this study, to extend our investigation and to increase our understanding of the precise components of HCV RC and the

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mechanisms of viral genome replication, we designed another comparative proteomic approach in which cells harboring genome-length bicistronic HCV RNA at the exponential growth phase (showing rapid replication of viral RNA) were compared with cells at the confluent-growth phase (showing poor replication of viral RNA). This strategy revealed that the chaperonin T-complex polypeptide (TCP1)-ring complex/chaperonin-containing TCP1 (TRiC/CCT) participates in HCV RNA replication and virion production possibly through an interaction between CCT5 (chaperonin-containing TCP1, subunit 5) and NS5B.

Results

CCT5 and Hsc70 are enriched in the DRM fraction containing the HCV RC

Recently, we analyzed the protein content of DRM fractions prepared from HCV subgenomic replicons and parental Huh-7 cells and identified 27 cellular proteins that were enriched in the DRM fraction prepared from the replicon cells (Hara et al., 2009). These were identified as factors that may be involved in the HCV RC and in viral replication. In fact, subsequent silencing of several genes coding for these proteins resulted in the inhibition of HCV RNA replication (Hara et al., 2009). However, it is likely that proteins unrelated to HCV replication are also included in the identified groups because long-term culture of the replicon cells under the selective pressure of G418 selects for a subpopulation of the parental cells and may induce changes in their protein expression profiles. Thus, to minimize interline differences in culture background, we further designed a comparative proteome analysis using a single cell line as follows.

HCV replication efficiency is dependent on the conditions of host cell growth. High cell density of the replicon culture has a reversible inhibitory effect on viral replication (Nelson and Tang, 2006; Pietschmann et al., 2001). Fig. 1A demonstrates that a high level of HCV RNA was detected in cells harboring the genome-length bicistronic HCV RNA, Con1 strain of genotype 1b (RCYM1) in the growth phase, whereas the RNA level declined sharply when the cells reached the stationary phase. We further compared the synthesis of HCV RNA in cell-free reaction mixtures containing the viral RC isolated from the RCYM1 cells at various cell densities (Fig. 1B). Replication activity was highest at the mid-log phase of cell growth (day 4 after seeding). By contrast, little or no RNA synthesis was observed under the confluent-growth cell culture (day 8), confirming the critical role of host cell growth conditions in the replication of the HCV genome.

Thus, to identify the host cell proteins required for HCV replication, we designed a two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)-based comparative proteomics analysis of RC-rich DRM fractions prepared from RCYM1 cells at the mid-log and confluent-growth phases. Protein spots that reproducibly showed a greater than 1.5-fold difference in the mid-log growth- and the confluent phases were excised and digested by trypsin or lysylendopeptidase. Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry (MS), which allows identification of the corresponding proteins in 9 cases (Table 1). Two increased spots that showed an increase in levels (their stereoscopic images are shown in Fig. 2A) were identified as CCT5 and Hsc70. CCT5, an epsilon subunit of chaperonin TRiC/CCT, is a 900-kDa toroid-shaped complex consisting of eight different subunits (Valpuesta et al., 2002; Yaffe et al., 1992). Hsc70, a member of the HSP70 family, is a 71-kDa heat shock cognate protein (Dworniczak and Mirault, 1987). Independent of the proteome analyses, DRM fractions and whole cell lysates were prepared from RCYM1 cells at two different growth phases (as above) and were analyzed by immunoblotting (Fig. 2B). Steady-state levels of CCT5 and Hsc70 were obviously higher in the DRM fraction prepared from the cells that were at the mid-log growth phase compared with those at the confluent phase. However, in the whole cell analyses, they were shown to be present at comparable levels during the two different growth phases. These results suggest that expression of CCT5 and Hsc70 is not enhanced in proliferating cells and that the enrichment of these proteins in the DRM fraction is possibly due to their post-translational modification. It should be noted that in the previous proteome analysis, CCT5 and other TRiC/CCT subunits, such as CCT1 and CCT2, were identified as proteins that were enriched in the DRM fraction prepared from subgenomic replicon-containing cells compared with that prepared from parental cells (Hara et al., 2009). We showed that CCT5 and CCT1 were enriched in the DRM fractions of cells transfected with the HCV genomic RNA derived from JFH-1 isolate as well as of subgenomic replicon cells (Fig. 2C).

TRiC/CCT participates in replication of the HCV genome

We investigated gain- and loss-of-functions of TRiC/CCT and Hsc70 with respect to the replication of HCV RNA. Seventy-two hours after RCYM1 cells were transfected with eight plasmids corresponding to each of the TRiC/CCT subunits, the level of HCV RNA in the cells (determined by quantitative RT-PCR) significantly increased to 2-fold that observed in the control cells. However, exogenous expression of Hsc70 in the RCYM1 cells showed no effect on the viral RNA (Fig. 3A). siRNAs targeted to CCT5 or Hsc70 and consisting of pools of three target-specific siRNAs or control nonspecific siRNAs were transfected

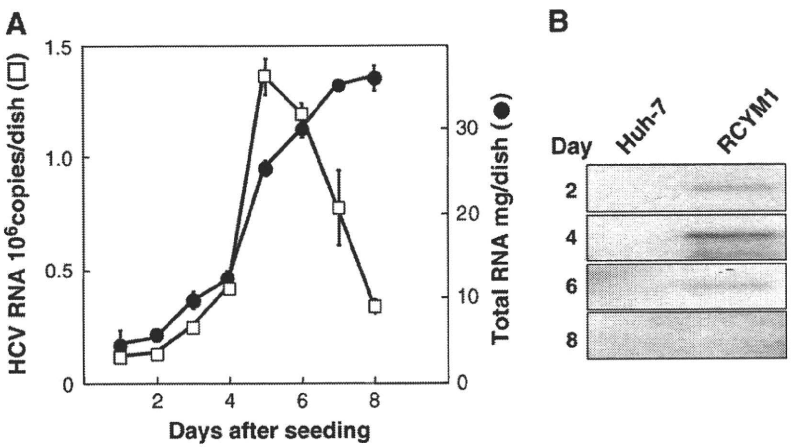

Fig. 1. Effect of cell growth on HCV RNA replication. (A) Measurement of HCV RNA (open squares) and total cellular RNA (closed circles) in RCYM1 cells at the time of harvest (days after seeding). (B) DRM fractions obtained from RCYM1 and parental Huh-7 cells harvested as indicated (day) were analyzed by cell-free RNA replication assay. RNA extracted from each sample was analyzed by agarose gel electrophoresis and autoradiograph.

Table 1
Selected cellular proteins that reproducibly increased and decreased in membrane fraction of RCYM1 cells at exponential growth phase.

Av. ratio	T-test	Coverage (%)	Protein name	Molecular function	GI
Increased proteins					
1.58	0.017	31	CCT5	Protein folding	33879913
1.54	0.005	35	HSPA8 (Hsc70)	Protein folding	24657660
Decreased proteins					
−1.95	0.028	44	Creatine kinase isozyme CK-B gene, exon 8	Energy pathway/metabolism	180568
−1.53	0.011	16	Chain C, Human Sirt2 Histone deacetylase	Cell cycle control	15826438
−2.14	0.001	33	Proteasome regulatory particle subunit p44S10	Metabolism	15341748
−1.71	0.004	21	Aldehyde dehydrogenase	Metabolism	178388
−1.85	0.004	40	Aminoacylase 1	Metabolism	12804328
−2.77	0.003	15	Eukaryotic translation initiation factor 3, subunit 3 gamma	Metabolism (translation regulator activity)	6685512
−2.43	0.014	20	Intraflagellar transport protein 74 homolog (Coiled-coil domain-containing protein 2)	Cell growth and/or maintenance	10439078

Three paired samples of RC-rich membrane fractions at the exponential- and confluent-growth phases of RCYM1 cultures were analyzed. The proteins representing a more than 1.5-fold increase or decrease (−) reproducibly and significantly are indicated.
Coverage (%): the ratio of the portion of protein sequence covered by matched peptides to the whole sequence.
GI: GenInfo Identifier number.

into RCYM1 cells. After 72 h, the HCV RNA level was reduced by 42% and 27% in the cells transfected with siRNAs against CCT5 and Hsc70, respectively, compared with controls (Fig. 3B). TRiC/CCT possibly interacts with Hsc70, and its complex formation contributes to increasing the efficiency of protein folding (Cuéllar et al., 2008). Our results suggest the involvement of TRiC/CCT and Hsc70 in the HCV life cycle. In particular, TRiC/CCT may play an important role in the replication of the viral genome.

To verify the specificity of the knockdown of CCT5 siRNA, we further synthesized two siRNAs targeted to different regions used in the above CCT5 siRNA and assessed their knockdown effect on HCV genome replication (Fig. 3C, upper panel). As expected, transfection of

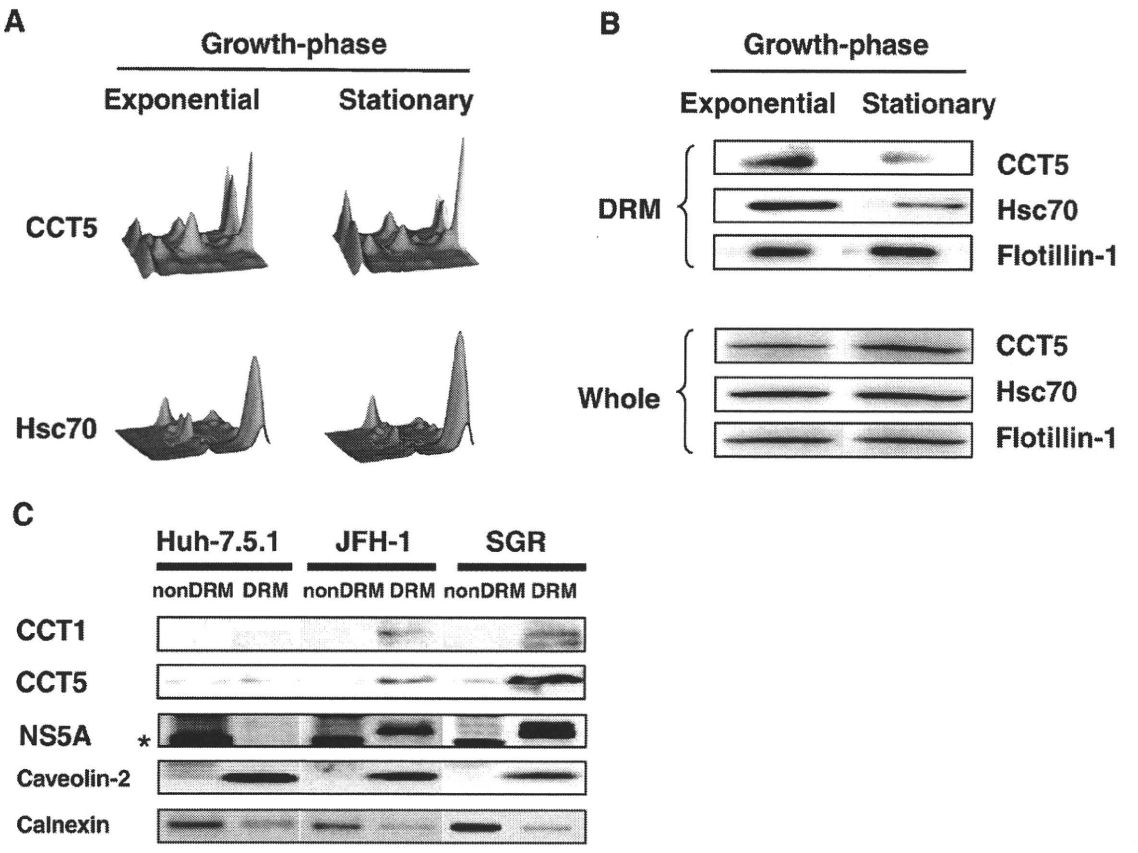

Fig. 2. Comparison of protein levels in DRM fractions prepared from RCYM1 cells at the exponential and stationary growth phases. (A) Three-dimensional images of CCT5 and Hsc70 analyzed by Ettan DIGE (GE Healthcare). Spots corresponding to CCT5/Hsc70 at exponential and stationary growth phases of the cells, respectively, are shown in green and red. (B) Equal amounts of protein in the DRM fractions prepared from RCYM1 cells at the exponential and stationary growth phases or corresponding whole cell lysates were analyzed by immunoblotting with Abs against CCT5, Hsc70 or flotillin-1. (C) Enrichment of CCT1 and CCT5 in the DRM fractions of HCV RNA replicating cells. Equal amounts of DRM or non-DRM fractions from full-length JFH-1 RNA transfected cells (JFH-1), subgenomic replicon cells (SGR) and parental Huh-7.5.1 cells were analyzed by immunoblotting with antibodies against CCT1, CCT5, NS5A, caveolin-2 or calnexin. *Non-specific bands.

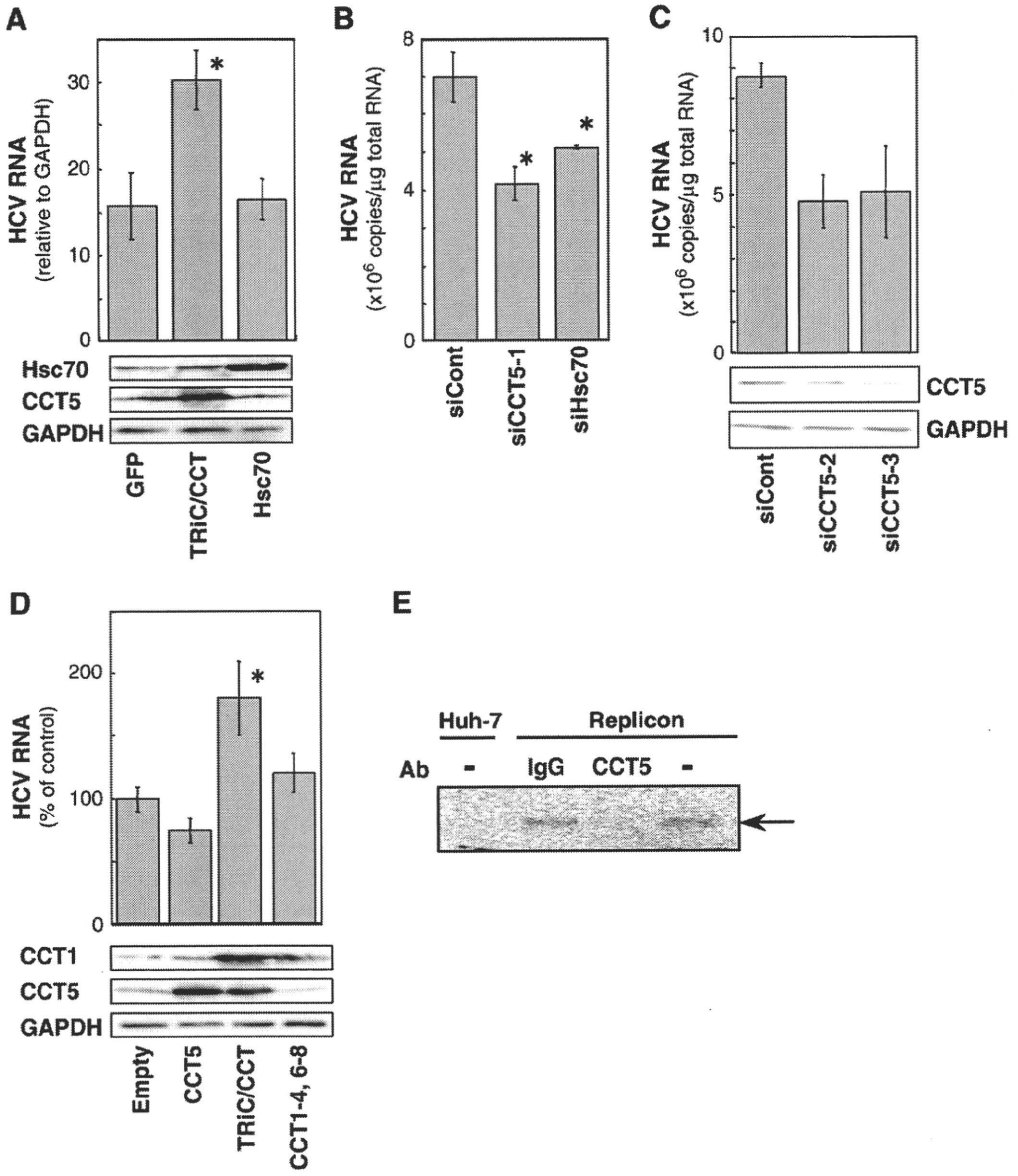

Fig. 3. Involvement of TRiC/CCT in HCV replication (A and D). Overexpression of all eight subunits of TRiC/CCT (TRiC/CCT); seven subunits, CCT1, 2, 3, 4, 6, 7, and 8 (CCT1–4, 6–8); subunit CCT5 only (CCT5); Hsc70; or control GFP in RCYM1 cells. HCV RNA levels were determined 48 h post-transfection (B and C). Knockdown of endogenous CCT5 or Hsc70 in RCYM1 cells, which were transfected with three types of siRNAs against CCT5 (siCCT5-1, -2, and -3), siRNA against Hsc70 (siHsc70), or control siRNA (siCont), and were harvested at 72 h post-transfection. siCCT5-1 and siHsc70 consisted of pools of three target-specific siRNAs. Immunoblotting for CCT1, CCT5, Hsc70 and GAPDH was performed (A, C and D; lower). (E) Cell-free de novo viral RNA synthesis assays were performed in the presence of anti-CCT5 Ab or control mouse IgG. Cytoplasmic fractions from SGR-N (replicon) and parental Huh-7 cells were used. An arrow indicates the synthesized HCV RNA. Error bars denote standard deviations with asterisks indicating statistical significance (*$P<0.01$).

RCYM1 cells with each CCT5 siRNA resulted in a reduction in viral RNA to a level of about 50% of that observed in cells treated with control siRNAs. Immunoblotting confirmed the efficient reduction in expression of endogenous CCT5 and the lack of cytotoxic effect exerted by the CCT5 siRNAs (Fig. 3C, middle and lower panels).

Having confirmed the upregulation of HCV RNA by ectopic expression of all the TRiC/CCT subunits, we further addressed the possibility that CCT5, independent of the complete TRiC/CCT complex, might have a role in promoting replication of HCV RNA. Transfection with either a CCT5 expression plasmid alone or with seven plasmids expressing all the TRiC/CCT subunits except CCT5 resulted in no or only a slight increase in the level of HCV RNA, indicating that all CCT subunits are required for HCV replication (Fig. 3D).

TRiC/CCT is generally known as a cytosolic chaperone (Valpuesta et al., 2002). However, it is enriched in the DRM fraction of HCV-replicating cells during the exponential growth phase (Fig. 2B). We used immunofluorescence staining to investigate whether TRiC/CCT is localized in the intracellular membrane compartments where replication of the viral genome occurs (Fig. 4). The de novo-synthesized RdRp was labeled by bromouridine triphosphate (BrUTP) incorporation in the presence of actinomycin D, and brominated nucleotides were detected with a specific antibody (Ab). Fluorescence staining in distinct speckles of various sizes was found in the cytoplasm of the HCV subgenomic replicon cells, whereas no signal was detected in the control cells, indicating that the observed BrUTP-incorporating RNA is mostly viral, newly synthesized viral RNA (Fig. 4A). Double immunofluorescence staining showed that a certain section of CCT5 co-distributed with the BrUTP-labeled RNA (Fig. 4A), which is known to co-exist with HCV NS proteins in viral replicating cells (Shi et al., 2003). We further observed that CCT5 was at least partially colocalized

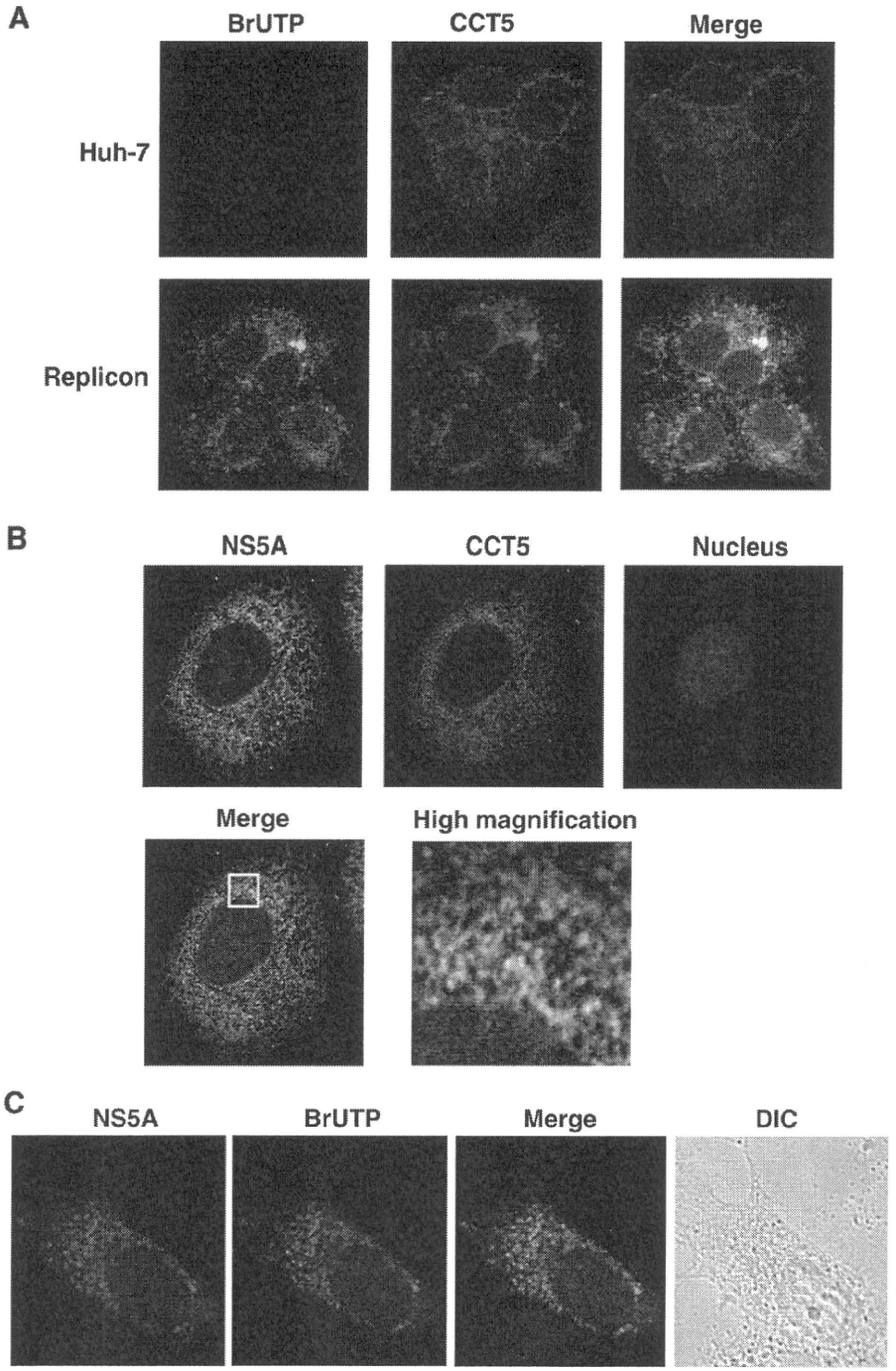

Fig. 4. Immunofluorescence analysis of CCT5 in SGR-N and Huh-7 cells (A) and HCVcc-infected cells (B). The primary Abs used were anti-CCT5 goat polyclonal Ab (red), anti-BrUTP monoclonal Ab (green), and anti-NS5A monoclonal Ab (green). Merged images of red and green signals (A) or of red, green and blue (nucleus) signals (B) are shown. The high magnification panel is an enlarged image of a white square of the merge panel. (C) Colocalization of NS5A protein with the viral RNA. The replicon cells were permeabilized with lysolecithin and labeled with BrUTP, followed by staining with anti-NS5A rabbit polyclonal Ab (red) and the anti-BrUTP monoclonal Ab (green). DIC, differential interference contrast.

with the viral NS protein in certain compartments sharing a dot-like structure in Huh-7 cells infected with HCV JFH-1 infectious HCV (HCVcc) derived from HCV genotype 2a (Fig. 4B) as well as in the replicon cells (data not shown). Fig. 4C indicated co-localization of BrUTP-labeled RNA with NS5A.

To further address the role of TRiC/CCT in HCV genome replication, we performed immunodepletion and in vitro replication analyses, which have been used for studying the genome replication of several viruses (Daikoku et al., 2006; Garcin et al., 1993; Liu et al., 2009). Cell extracts prepared from the HCV-replicating cells were reacted with either a mouse monoclonal Ab against CCT5 or mouse IgG derived from preimmune serum, followed by cell-free synthesis of HCV RNA. Fig. 3E shows that treatment with anti-CCT5 Ab inhibited viral RNA synthesis, whereas the control IgG did not affect the process, suggesting that TRiC/CCT participates directly in HCV RNA replication.

CCT5 interacts with HCV NS5B

The genome replication machinery of HCV is a membrane-associated complex composed of multiple factors including viral NS proteins. Given the involvement of TRiC/CCT in HCV RNA synthesis, we next examined its possible interaction with HCV NS proteins. A first attempt to immunoprecipitate the viral proteins with antibodies against TRiC/CCT subunits in the replicon cells was unsuccessful (data not shown), suggesting that endogenous levels of TRiC/CCT is not sufficient to pull out NS5B. Next, dual (myc/FLAG)-tagged NS3, NS5A, or NS5B proteins derived from the genotype 1b NIHJ1 strain were co-expressed with CCT5 in Huh-7 cells and then subjected to two-step immunoprecipitation with anti-myc and anti-FLAG Abs (Ichimura et al., 2005; Shirakura et al., 2007). An empty plasmid was used as a negative control in the analyses. As shown in Fig. 5A, CCT5 specifically interacted with NS5B. Little or no interaction was found between CCT5 and NS3 or NS5A. To determine the NS5B region required for the interaction with CCT5, various deletion mutants of HA-NS5B were constructed and their interactions with CCT5 were analyzed as described above. CCT5 was shown to be coimmunoprecipitated with either a full-length NS5B (aa 1–591), an N-terminal deletion (aa 71–591) or a C-terminal deletion (aa 1–570), but not with deletions aa 215–591 or aa 320–591 (Fig. 5B), suggesting that aa 71–214 of NS5B are important for its interaction with CCT5.

Knockdown of CCT5 results in the reduction of propagation of infectious HCV

We further examined whether the knockdown of CCT5 would abrogate the production of infectious HCV (HCVcc), derived from JFH-1 (Fig. 6). At 72 h post-transfection with each CCT5 siRNA, HCV RNA

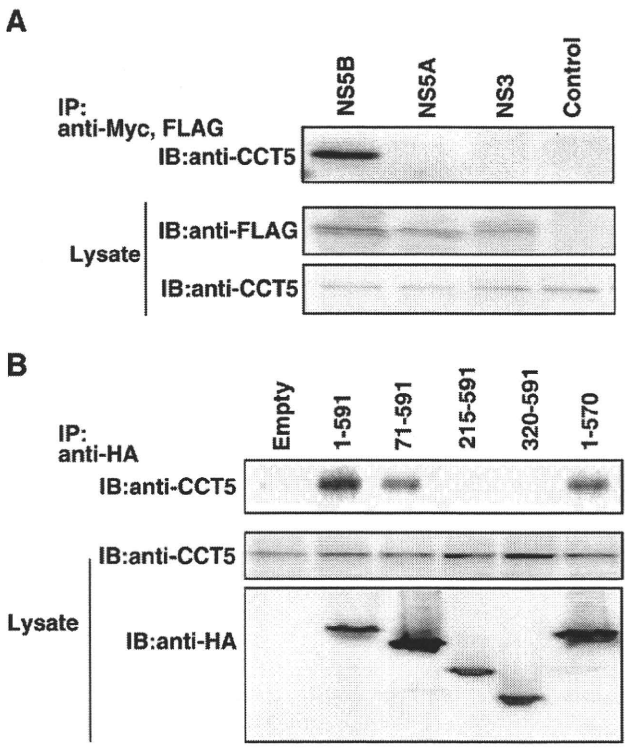

Fig. 5. CCT5 interacts with HCV NS5B. (A) CCT5 was co-expressed with MEF-tagged-NS5B, -NS5A, or -NS3 protein of strain NIHJ1 in cells, followed by two-step immunoprecipitation (IP) with anti-FLAG and anti-myc Abs. Immunoprecipitates were subjected to immunoblotting with anti-CCT5 Ab (IB). (B) Full-length NS5B (1–591) or its deletions (71–591, 215–591, 320–591, 1–570) along with a HA tag were co-expressed with CCT5. IP and IB were performed as described above.

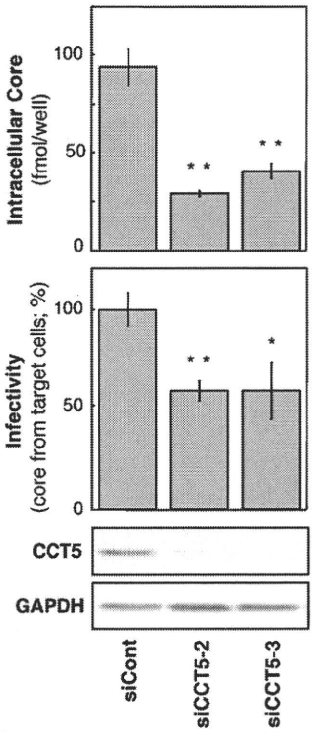

Fig. 6. Knockdown of endogenous CCT5 in HCVcc-infected cells. The cells were transfected with siRNAs against CCT5 (siCCT5-2, -3) or with control siRNAs (siCont). At 72 h post-transfection, the viral core protein levels in cells were determined (upper panel). Collected culture supernatants were inoculated into naïve Huh7.5.1 cells and intracellular core proteins were determined at 72 h post-infection (middle panel). Cells transfected with siRNAs were analyzed by immunoblotting with anti-CCT5 or anti-GAPDH Ab (lower panel). Error bars denote standard deviations with asterisks indicating statistical significance (*$P<0.05$; **$P<0.01$).

levels in Huh-7 cells infected with HCVcc were reduced by 25–35% compared with controls. Accordingly, virion production from CCT5 siRNA-transfected cultures was significantly decreased, as determined by intracellular HCV core protein levels at 72 h after the infection of naïve cells with culture supernatants taken from transfected cells. These results demonstrate that reduction of the HCV RNA replication by siRNA-mediated knockdown of CCT5 results in reduction of the propagation of the infectious virus.

Discussion

The chaperone-assisted protein-folding pathway is a process in living cells that results from coordinated interactions between multiple proteins that often form multi-component complexes. Several steps in the viral life cycle, such as protein processing, genome replication, and viral assembly, are regulated by cellular chaperones. Hsp90, one of the most abundant proteins in unstressed cells, has been implicated in HCV RNA replication (Nakagawa et al., 2007; Okamoto et al., 2006, 2008; Taguwa et al., 2008, 2009; Ujino et al., 2009). FKBP8, a member of the FKBP506-binding protein family, and hB-ind1, human butyrate-induced transcript 1, play key roles through their interaction with HCV NS5A and Hsp90 (Okamoto et al., 2006, 2008; Taguwa et al., 2008, 2009). Hsp90 has also been implicated in viral enzymatic activities including those of the influenza virus (Momose et al., 2002; Naito et al., 2007), herpes simplex virus (Burch and Weller, 2005), Flock house virus (Kampmueller and Miller, 2005), and hepatitis B virus (Hu et al., 2004).

In our former study, comparative proteome analyses of the viral RC-rich DRM fractions prepared from subgenomic replicon cells and Huh-7 cells were carried out to identify host factors involved

in HCV replication (Hara et al., 2009). We extended the proteomics by modifying our protocol of the analysis to reduce the interline differences in culture background and analyzed the DRM samples derived from the mid-log and confluent-growth phases of single cell line. Here, we identified two proteins, CCT5 and Hsc70, showing an increase in levels at the mid-log growth phase. Although CCT5 was also identified in the former study as expected, Hsc70 was not included in the list of proteins identified in the study (Hara et al., 2009). This difference may be due to the use of cells carrying the full-length replicon RNA in this study.

In this study, we demonstrated that TRiC/CCT participates in HCV RNA replication and virion production possibly through its interaction with NS5B. TRiC/CCT is a group II chaperonin that assists in protein folding in eukaryotic cells and forms a double-ring-like hexadecamer complex. Although relatively little is known about its function compared with that of the group I chaperonins such as bacterial GroEL, several mammalian proteins whose folding is mediated by TRiC/CCT have been identified, such as actin, tubulin, and von Hippel–Lindau tumor suppressor protein (Farr et al., 1997; Feldman et al., 2003; Frydman and Hartl, 1996; Meyer et al., 2003; Tian et al., 1995). With regard to viral proteins, the Epstein–Barr virus nuclear antigen, HBV capsid protein, and p4 of M-PMV have been identified as TRiC/CCT-interacting proteins (Yam et al., 2008). However, the functional significance of their interactions in the viral life cycles has yet to be determined. Here we demonstrated that the reduction in CCT5 expression in HCV replicon cells and in virus-infected cells inhibits HCV RNA replication (Figs. 3B and C) and virus production (Fig. 6) respectively. Gain-of-function was also shown by co-transfection of the replicon cells with eight constructs corresponding to all the TRiC/CCT subunits (Figs. 3A and D).

A recent study of the three-dimensional structure of the TRiC/CCT and Hsc70 complex has demonstrated that the apical domain of the CCT2 (CCT-beta) subunit is involved in the interaction with Hsc70 (Cuéllar et al., 2008). The complex formation created by the TRiC/CCT and Hsc70 interaction may promote higher efficiency in the folding of certain proteins (Cuéllar et al., 2008). In our comparative proteome analyses, both CCT subunits and Hsc70 were enriched in the HCV RC-rich membrane fraction of the replicon cells that showed high viral replication activity (Fig. 2B). Transfection of Hsc70 siRNA into the replicon cells moderately inhibited viral RNA replication (Fig. 3B). However, upregulation of HCV replication was not observed by ectopic expression of Hsc70 (Fig. 3A), and little or no interaction was observed between Hsc70 and HCV NS proteins in the co-immunoprecipitation analysis (data not shown). Thus, it is likely that TRiC/CCT acts as a regulator of HCV replication through participating in the de novo folding of NS5B RdRp, and Hsc70 might serve to assist in folding through its interaction with TRiC/CCT. It was recently reported that Hsc70 is associated with HCV particles and modulates the viral infectivity (Parent et al., 2009). Here we showed an additional role of Hsc70 in the HCV life cycle.

HCV genomic single-stranded RNA serves as a template for the synthesis of the full-length minus strand that is used for the overproduction of the virus-specific genomic RNA. NS5B RdRp is a single subunit catalytic component of the viral replication machinery responsible for both of these processes. It is known that the in vitro RdRp activity of recombinant NS5B expressed in and purified from insect cells and *Escherichia coli* is low in many cases. This could be due to the lack of a suitable cellular environment for favorable RdRp activity, although the particular conformational features dependent on the viral isolates may also be involved (Lohmann et al., 1997; Weng et al., 2009). In fact, besides interacting with HCV NS proteins, NS5B has been reported to interact with several host cell proteins. For example, human vesicle-associated membrane protein-associated protein subtype A (VAP-A) and subtype B (VAP-B), which are involved in the regulation of membrane trafficking, lipid transport and metabolism, and the unfolded protein response, interact with NS5B and NS5A and participate in HCV replication (Hamamoto et al., 2005). Recently, VAP-C, a splicing variant of VAP-B, was found to act as a negative regulator of viral replication through its interaction with NS5B but not with VAP-A (Kukihara et al., 2009). Cyclophilin A and B, peptidyl-prolyl isomerases that facilitate protein folding by catalyzing the *cis–trans* interconversion of peptide bonds at proline residues, play a role in stimulating HCV RNA synthesis through interaction with NS5B (Liu et al., 2009; Watashi et al., 2005). SNARE-like protein (Tu et al., 1999), eIF4AII (Kyono et al., 2002), protein kinase C-related kinase 2 (Kim et al., 2004), nucleolin (Kim et al., 2004; Hirano et al., 2003; Shimakami et al., 2006), and p68 (Goh et al., 2004) are also known to associate with NS5B and are possibly involved in HCV RNA replication.

We found that the aa 71–214 region in NS5B is important for interaction with TRiC/CCT. The catalytic domain of HCV RdRp has a "right-hand" configuration similar to other viral polymerases, such as HIV-1 reverse transcriptase (Huang et al., 1998) and poliovirus RdRp (Hansen et al., 1997), and is divided into the fingers, palm, and thumb functional subdomains (Lohmann et al., 2000). The region required for the interaction with TRiC/CCT has been mapped in a part of the fingers and palm domains of NS5B RdRp. To address how TRiC/CCT assists in the correct folding or disaggregation of NS5B through their interaction, leading to the formation of a functional RdRp, work based on an in vitro reconstitution system using purified proteins is under way. As all the TRiC/CCT subunits possess essentially identical ATPase domains, their protein-recognition regions are apparently divergent, allowing for substrate-binding specificity. It has recently been reported that TRiC/CCT interacts with the PB2 subunit of the influenza virus RNA polymerase complex and TRiC/CCT binding site is located in the central region of PB2, suggesting involvement of TRiC/CCT in the influenza virus life cycle (Fislová et al., 2010). Eukaryotic RNA polymerase subunit has also been identified as a binding partner of TRiC/CCT from interactome analysis (Yam et al., 2008). It would be interesting to examine how conserved the mechanisms of TRiC/CCT action that result in enhanced replication are among RNA polymerases.

The recruitment of a chaperonin by viral NS proteins may be important for understanding regulation of the viral genome replication. In this study, we demonstrated the involvement of TRiC/CCT in HCV RNA replication possibly through its interaction between TRiC/CCT and HCV NS5B. Although possible interaction of subunit CCT5 with NS5B was shown, considering involvement of whole TRiC/CCT complex in its chaperonin function, whether CCT5 directly interacts with NS5B is unclear. Further detailed studies are needed to make clear the manner of TRiC/CCT-NS5B interaction. NS5B RdRp is one of the main targets for HCV drug discovery. The search for NS5B inhibitors has resulted in the identification of several binding sites on NS5B, such as the domain adjacent to the active site and the allosteric GTP site (De Francesco and Migliaccio, 2005; Laporte et al., 2008). The findings obtained here suggest that disturbing the interaction between NS5B and TRiC/CCT may be a novel approach for an antiviral chemotherapeutic strategy.

Materials and methods

Cell culture, transfection, and infection

Human hepatoma Huh-7 and Huh-7.5.1 cells (kindly provided by Francis V. Chisari from The Scripps Research Institute) and human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Huh-7-derived SGR-N (Shi et al., 2003) and RCYM1 (Murakami et al., 2006) cells, which possess subgenomic replicon RNA from the HCV-N strain (Guo et al., 2001; Ikeda et al., 2002) and genome-length HCV RNA from the Con 1 strain (Pietschmann et al., 2002), were cultured in the above medium in the presence of 1 mg/ml G418. Cells were transfected with plasmid DNAs using FuGENE transfection reagents

(Roche Diagnostics, Tokyo, Japan). Culture media from Huh-7 cells transfected with in vitro-transcribed RNA corresponding to the full-length HCV RNA derived from the JFH-1 strain (Wakita et al., 2005) were collected, concentrated, and used for the infection assay (Aizaki et al., 2008).

Ab

Primary Abs used in this study were mouse monoclonal Abs against FLAG (Sigma-Aldrich, St. Louis, MO), c-myc (Sigma-Aldrich), CCT5 (Abnova Corporation, Taipei City, Taiwan), flotillin-1 (BD Biosciences, San Jose, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon, Temecula, CA), BrdU (Caltag, CA) and HCV NS5A (Austral Biologicals, San Ramon, CA), a rabbit polyclonal Ab against hemagglutinin (HA; Sigma-Aldrich), a sheep polyclonal Ab against bromodeoxyuridine (Biodesign International, Saco, ME), and goat polyclonal Abs against the individual subunits of CCT (Santa Cruz Biotechnology, Santa Cruz, CA) and Hsc70 (Santa Cruz Biotechnology). Anti Hsc70 and CCT5 monoclonal rat Abs were obtained from Abcam (Tokyo, Japan) and AbD serotec (Oxford, UK). Rabbit polyclonal antibody to NS5A was described previously (Hamamoto et al., 2005). Anti NS5B monoclonal Ab was kindly provided by D. Moradpour (Centre Hospitalier Universitaire Vaudois, University of Lausanne; Moradpour et al., 2002).

Plasmids

To generate expression plasmids for the NS proteins with dual epitope tags, DNA fragments encoding the NS3, NS5A, or NS5B proteins were amplified from HCV strain NIHJ1 (Aizaki et al., 1998) by PCR and cloned into the EcoRI–EcoRV sites of pcDNA3-MEF, which includes the MEF tag cassette containing the *myc* tag, TEV protease cleavage site, and FLAG tag sequences (Ichimura et al., 2005; Shirakura et al., 2007). To create a series of NS5B truncation mutants, each fragment was amplified by PCR and cloned into the EcoRI–XhoI site of pCMV-HA (Clontech, Mountain View, CA). To generate expression plasmids for the individual CCT subunits, cDNA fragments encoding human CCT1 through CCT8 were amplified from the total cellular RNA by RT-PCR and then cloned into the SmaI site of pCAGGS (Niwa et al., 1991). All PCR products were confirmed by nucleotide sequencing.

Proteome analysis

RC-rich membrane fractions from the cells were isolated as described previously (Aizaki et al., 2004). Briefly, cells were lysed in hypotonic buffer. After removing the nuclei, the supernatants were mixed with 70% sucrose, overlaid with 55% and 10% sucrose, and centrifuged at 38,000 rpm for 14 h. Proteins from the membrane fractions were then analyzed by 2D-DIGE as described previously (Hara et al., 2009). Briefly, protein samples were resolved in protein solubilization buffer (Bio-Rad Laboratories, Tokyo, Japan) and washed with pH adjustment buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl [pH 10.0]), before being labeled with fluorescent dyes; the dyes used were Cy3 for RCYM1 cells samples taken at the exponential growth phase, Cy5 for cells samples taken at the confluent phase, and Cy2 for a protein standard containing equal amounts of both cell samples. Aliquots of the labeled samples were pooled and applied to Immobiline DryStrip (GE Healthcare, Tokyo, Japan) for first-dimension separation and to 12.5% polyacrylamide gels for second-dimension separation. Images of the 2-D gels were captured on a Typhoon scanner (GE Healthcare), and analyzed quantitatively using DeCyder v5.0 software (GE Healthcare). Samples were analyzed in triplicate as independent cultures and the Student's *t*-test was applied using the DeCyder biological variation analysis module to validate the significance of the differences in spot intensity detected between the samples.

In vitro RNA replication assay

In vitro replication of HCV RNA was performed as described previously (Hamamoto et al., 2005). Briefly, cytoplasmic fractions of subgenomic replicon cells were treated with 1% NP-40 at 4 °C for 1 h, followed by being incubated with 1 mM of ATP, GTP, and UTP; 10 μM CTP; [^{32}P]CTP (1 MBq; 15 TBq/mmol); 10 μg/ml actinomycin D; and 800 U/ml RNase inhibitor (Promega, Madison, WI) for 4 h at 30 °C. RNA was extracted from the total mixture by using TRI Reagent (Molecular Research Center, Cincinnati, OH). The RNA was precipitated, eluted in 10 μl of RNase-free water, and analyzed by 1% formaldehyde-agarose gel electrophoresis. For the immunodepletion assay, the cytoplasmic fractions were incubated with anti-CCT5 Ab in the presence of NP-40 for 4 h before NTP incorporation.

MALDI-TOF MS analysis

Target spots were cut and collected from gels under UV luminescence and rechecked with Typhoon scanner. The spot gels of the target proteins were subjected to in-gel trypsin digestion and analyzed by MALDI-TOF MS meter (Voyager-DE STR, Applied Biosystems, Tokyo, Japan) as described previously (Yanagida et al., 2000). All proteins were identified by peptide mass fingerprinting.

Immunoblot analysis and immunoprecipitation

Immunoblot analysis was performed essentially as described previously (Aizaki et al., 2004). The membrane was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). For immunoprecipitation, cells transfected with plasmids expressing epitope-tagged HCV protein or CCT5 were lysed and then subjected to two-step precipitations with anti-myc and anti-FLAG Abs according to the procedures described previously (Ichimura et al., 2005). In some experiments, HA-tagged full-length NS5B (aa 1–591) or its deletion mutants (aa 71–591, 215–591, 320–591, 1–570) were co-expressed with CCT5 in cells, followed by single-step immunoprecipitation and immunoblotting.

Immunofluorescence staining

Cell permeabilization with lysolecithin and detection of de novo-synthesized viral RNA was performed as described previously (Shi et al., 2003). Briefly, Huh-7 cells were plated on 8-well chamber slides at a density of 5×10^4 cells per well. Cells were incubated with actinomycin D (5 μg/μl) for 1 h and were washed twice with serum-free medium, before being incubated for 10 min on ice. The cells were then incubated in a transcription buffer containing 0.5 mM BrUTP for 30 min. The cells were fixed in 4% formaldehyde for 20 min and then incubated for 15 min in 0.1% Triton X-100 in phosphate-buffered saline (PBS). Primary Abs were diluted in 5% bovine serum albumin in PBS and were incubated with the cells for 1 h. After washing with PBS, fluorescein-conjugated secondary Abs (Jackson Immunoresearch Laboratories, West Grove, PA) were added to the cells at a 1:200 dilution for 1 h. The slides were then washed with PBS and mounted in ProLong Antifade (Molecular Probes, Eugene, OR). Confocal microscopy was performed on a Zeiss Confocal Laser Scanning Microscope LSM 510 (Carl Zeiss MicroImaging, Thornwood, NY).

RNA interference

Small interfering RNAs (siRNAs) targeted to CCT5 or Hsc70 and scrambled negative control siRNAs were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Cells were plated on a 24-well plate with