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Gene expression profiling of human hepatoma cells in three-dimensional culture

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Abstract

It is generally difficult to maintain physiological functions and properties of human liver-derived cells in conventional monolayer (2D) cultures. In this study, we established a three-dimensional (3D) culture of human hepatoma HepG2 using thermoreversible gelation polymer and used cDNA microarray analysis to identify genes expressing difference in 2D and 3D cultures. Among the subset of genes with≧2-fold difference in expression was a number of regulators for cell growth, differentiation, transport and lipid metabolism. IGFBP3, which is known to down-regulate cell growth and to induce apoptosis, was identified as one of the remarkably up-regulated genes in the 3D culture. Further transcriptome analysis demonstrated altered gene expression in the cells over-expressing IGFBP3. This approach will enable us to expand our research, thereby improving our understanding of regulatory mechanisms underlying cell growth in the 3D settings.

〈**Key words**〉 HepG2, Insulin-like growth factor binding protein 3 (IGFBP3), three-dimensional culture, Thermo-reversible Gelation Polymer (TGP)

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●特集　生体膜関連シンポジウム「脂質低下療法時代の生体膜研究」

生体膜脂質のC型肝炎ウイルス生活環における役割

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Critical Role of Membrane–associated Lipid in the Life Cycle of the Hepatitis C Virus

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Systems of subgenomic replicon and recombinant infectious hepatitis C virus (HCV) have been established in 1999 and 2005, and virological techniques are able to be applied to the HCV research, especially regarding molecular mechanisms on replication and virion assembly, respectively. We showed that HCV active replication complex contains membrane structures, characteristic of lipid rafts. We also recently demonstrated an important role of cholesterol and sphingolipid in HCV infection and virion maturation. Finally, inhibitors of cellular cholesterol and sphingolipid biosynthetic pathway efficiently block virion production. Association of hepatitis C virus with lipid can be also a source of new antiviral therapies.

Key words : hepatitis C virus／life cycle／subgenomic replicon／cholesterol／sphingolipid

1. はじめに

C型肝炎ウイルス（HCV）感染者は日本で約200万人，世界中で1億7000万人にのぼる[1]とされ，その多くが10～30年という長期間を経て慢性肝炎から肝硬変へと進行し，高率に肝細胞癌を発症している[2]．現在，HCV感染症に対する主要な治療法はインターフェロン（IFN）とリバビリンによる併用療法であるが，有効となるのは半数の患者にすぎない．より有効な治療法の開発が望まれているが，HCVには効率の良いウイルス培養系が存在しなかったため，HCVの基礎研究はウイルス遺伝子の発現産物の機能解析を中心に進んだ．また，実験用の感染小動物が存在しないため，HCVのウイルス学的な解析はチンパンジーを用いた感染実験に頼るしか無く，倫理的な問題やコストの面からも安易にできる実験ではなかった．このような状況がHCVの基礎研究の妨げになり，抗ウイルス薬やワクチンの開発が遅れてきた．しかし，1999年に培養細胞で自律複製する構造領域を欠くサブゲノムレプリコンが開発され[3]，これを皮切りにHCVの複製に関する研究が精力的に進められてきた．さらに，劇症肝炎患者から単離されたJFH-1株のゲノムRNAを肝癌細胞由来のHuh–7細胞に導入することにより，感染性ウイルス粒子を培養細胞で作製する技術が2005年に確立された[4]．これにより，HCVの生活環のすべてを再現可能な実験系が確立したことになり，HCV研究を急速に加速させた．

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2. HCVの生活環

推定されているHCVの生活環をFig. 1に示す．HCVがレセプターを介して肝細胞に感染（吸着Attachment，侵入Entry）し，粒子よりウイルスRNAが放出され（脱核Cytoplasmic release），これがメッ

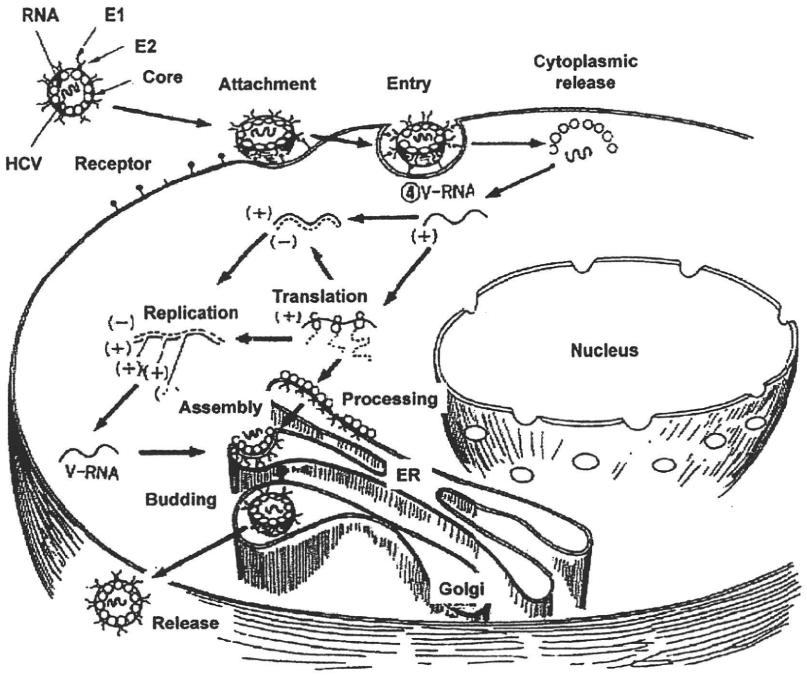

Fig. 1　HCV life cycle [18].

センジャーRNAとして働き，このRNAの5'非翻訳領域に存在するIRESから翻訳（Translation）が開始され大きな前駆体蛋白が合成される．この前駆体蛋白は，細胞のシグナラーゼによってウイルス粒子を形成する構造蛋白であるコア蛋白と2つのエンベロープ蛋白E1，E2がプロセス（Processing）される．また，ウイルス自身がコードするプロテアーゼによって，プロテアーゼ，ヘリカーゼ，RNA依存性RNAポリメラーゼ（RdRp）などウイルスの複製に必須な非構造蛋白がプロセスされる．ウイルスにコードされた酵素や宿主因子によってゲノムRNAからマイナス鎖RNAが転写され，複製複合体が形成される．これを基にしてプラス鎖RNAが合成され（複製Replication），ウイルスRNAやmRNAとして働く．ウイルスRNAがコア蛋白と結合してヌクレオカプシドを形成し，さらにエンベロープ蛋白が邂逅して小胞体（ER）でウイルス粒子が成熟し（出芽Assembly），トランスゴルジを通り細胞膜に達して細胞外へ放出（Release）されるものと考えられている．以上のようなHCV生活環のうち多くのステップでウイルスは細胞のER，ゴルジ体，形質膜といった生体膜を使っていると推定されている．

3. 生体膜脂質のウイルスゲノム複製における役割

ウイルスゲノム複製においてNS5B遺伝子のコードするRdRpが中心的な役割を担っているものと考えられている．しかしながら，強制発現させたRdRpを精製し解析したところ，その活性は鋳型特異性がなく，複製産物の長さは鋳型と異なった．一般的に，鋳型特異的なRNA合成には細胞因子や他のNS蛋白が必要と考えられている[5]．以上のことから，HCV複製の研究にはNS5Bだけでなく，他のNS蛋白や宿主因子が結合した複製複合体を維持した上での解析が重要ということが考えられる．従って，HCVレプリコンシステムはHCVゲノムの複製機構を解析する上で非常に有効と期待された．1999年，Bartenschlagerらは，本来HCVゲノムの中でウイルス粒子を形成する構造タンパク質領域を薬剤耐性遺伝子に置き換え，その下流に，より強力にHCVゲノムの内部から翻訳させる働きを有するencepharomyocarditis virus（EMCV）のIRESを挿入したRNAレプリコンを作製した[3]．このRNAをトランスフェクトした細胞を薬剤存在下で培養することで，自律複製するHCV遺伝子配列を獲得したHCVゲノムと，更にこのHCV遺伝子が複製しうる細胞を選択することを目指した．そして，このようなHCVのRNAレプリコンの複製を許容できる細胞がトランスフェクトしたヒト肝細胞癌由来Huh7細胞の一部から得られ，これによりHCVで初めてタンパク質レベルでウイルスの複製・増殖を解析できる系が確立された．

筆者らはこのレプリコン細胞内で複製しているHCV遺伝子を観察したいと考え，レプリコン細胞を

用いて，アクチノマイシンD処理して細胞内のDNA依存性RNAポリメラーゼを抑えた上で，5-bromouridine 5'-triphosphate（BrUTP）を細胞に導入し，免疫組織染色で観察した[6]．BrUTPが取り込まれた新規に合成されたHCV RNAはレプリコン細胞の核周辺の細胞質に斑点状の構造物として認められ，これらはNSタンパク質と共局在した．レプリコン細胞を電子顕微鏡で観察すると「membranous web」と呼ばれる小胞様構造物が認められることが報告されており[7]，HCVの全ての構造，非構造蛋白を強制発現させても同様の膜変化が生じることが知られている．このような変化は，HCVが感染したチンパンジーの肝細胞の電顕観察でスポンジ状の形態変化として報告されている[8]．以上のことから，HCVの複製複合体は感染細胞のmembranous webに存在しているものと思われる．

次に，生化学的手法を用いて，複製活性を維持したままのHCV複製複合体を粗精製し解析することを目指した[9]．細胞を低浸透圧液に溶解し，ホモジナイズを行った後，核画分を除き，ショ糖密度勾配法で膜とその他の細胞質成分に分画した．それぞれの画分について，多糖体でできたダイアフロー限外濾過膜を用いて限外濾過を行い，低分子量のタンパク質は除去した．HCV RNAとNSタンパク質は膜画分に検出された．lysateをNonidet P-40（NP-40）やTriton X-100（TX-100）などの非イオン性界面活性剤で処理した後，同様に分画したところ，HCV RNAとNSタンパク質の大部分は界面活性剤不溶性画分（DRM）に残った．それぞれの画分に標識化合物（CTP）を加え，この取り込みを指標にしたHCV RNA複製活性測定を行ったところ，活性はDRMにのみ検出された．以上のことから，このDRMに複製活性を保持したHCV複製複合体が存在することが判明した．界面活性剤可溶性画分（DSM）にもNSタンパク質は認められたが，限外濾過を行ったところ，検出されなくなったことから，このNSタンパク質はHCV複製複合体を形成していないと考えられた．以上のように，HCV複製複合体がDRMに検出されたことから，HCV複製複合体が脂質ラフトと結合している可能性が示唆された．そこでこのことを確かめるために，脂質ラフトの構成成分であるコレステロールを除去する働きのあるサポニン（pore-forming agent）で処理したところ，NSタンパク質は脂質ラフトのマーカーであるカベオリン2と共にDRMからDSMへと移行した．更に，HMG-CoAレダクターゼ阻害剤のロバスタチンでレプリコン細胞内のコレステロール合成を抑制するとHCV RNA複製効率も落ちることから，脂質ラフトがHCV複製複合体と結合し，

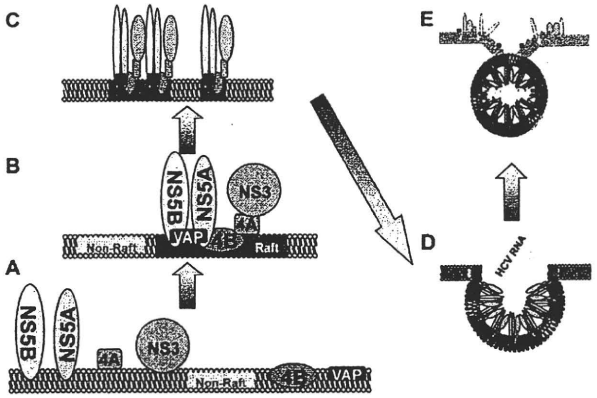

Fig. 2　Model of HCV replication[9].

HCV複製において重要な役割を果たしている可能性が示唆された[9]．また，NS蛋白が脂質ラフトに結合するのを抑制することで，各種スフィンゴ脂質合成阻害剤がウイルス複製を抑えるという報告があり，脂質ラフトの存在する膜上で複製が起こるという仮説が支持された[10]．

以上のように，HCVは細胞の生体膜上の小胞内で複製複合体を形成し，複製するものと考えられている．脂質ラフトは細胞膜上にスフィンゴ脂質とコレステロールに富んだ微小領域を示す．この脂質ラフトは，膜表面上をイカダのように漂いながら，ラフト同士が結合して島状のものになったり，小胞を形成したりと，ダイナミックに変化しながら，ラフトに結合するタンパク質の濃縮や細胞内輸送，シグナルトランスダクション，脂質代謝を担っていると考えられる[11]．また，脂質ラフトはインフルエンザウイルスの集合・出芽，ヒト免疫不全ウイルスの集合・出芽や侵入，エボラウイルスの集合，コクサッキーウイルスA9の侵入，HTLV-1の膜融合や集合，マウス白血病ウイルスの侵入，麻疹ウイルスの集合，センダイウイルスの集合，RSウイルスの集合，マーブルグウイルス，ロタウイルスの集合，ヒト単純ヘルペスウイルスの集合や侵入，エコーウイルス11の侵入，などの多くのウイルスの侵入や粒子形成に重要な役割を果たしていることが報告されている．しかしながら，ウイルスゲノム複製に影響を与えることはHCV研究で初めて示された[9]．

Fig. 2にHCV複製複合体形成モデルを示す．HCVNS蛋白はERで合成され，NS4Bは膜に，NS5Aはその5末端で，NS5Bはその3末端で膜にアンカーしている（A）．HCVNS蛋白はゴルジ体に輸送され，HCVNS蛋白同士で結合する．また，細胞内膜タンパク質の一つで，細胞内膜輸送に関わっていると考えられているthe human homologue of the 33-kDa vesi-

cle–associated membrane protein–associated protein（VAP–A）はそのN末端でNS5Bと，中央部のコイルドコイル領域でNS5Aと結合する[12]．NS5Aは脂質ラフトと弱く結合し，NS4Bは強く結合する．以上から，NS4Bが中心となって，hVAP–33やNS5Aと共に，他のNS蛋白を脂質ラフト上に誘導・固定する役割を担っているものと思われる（B）．一般的に，脂質ラフトは自由に膜上を移動し，集散を繰り返しているものと考えられている．しかしながら，NS4Bのように互いに結合する蛋白が乗っている場合，一度結合した脂質ラフト同士は安定化し，島状に次第に大きくなり，その過程で特定の蛋白を集積させる性格がある（C）．さらに，膜上の蛋白同士が結合するエネルギーにより，膜は小胞を形成するようになる（D）．既に，NS4B蛋白単独でもこの小胞構造を取ることが報告されている．ここにHCV RNAが取り込まれることにより，複製複合体を作り，複製が始まるものと考えられる（E）．以上のように，脂質ラフトはNS蛋白を集積させ，結合体を形成させるだけでなく，小胞構造をとり，膜に包まれたHCV複製の場を提供する役割があるものと想定されている．

筆者らはレプリコン細胞株からHCV複製複合体を含む画分を上記の方法で複製活性を維持したまま抽出しその構造を解析した[9]．鎖特異的PCRを用い，複製複合体中のプラス，マイナス鎖RNAのコピー数について調べたところ，マイナス鎖RNA 1に対してプラス鎖RNA10であった．分画を1% NP–40，4℃で処理後，RNA分解酵素やプロテアーゼで処理してもHCV RNAやNSタンパク質は分解されなかったが，脂質ラフトが破壊されるような強い条件（1% TX–100，37℃）で処理したところ，HCV RNAやNSタンパク質はRNA分解酵素やプロテアーゼ感受性に変化した．このことから，HCV複製複合体は脂質ラフトを含む膜小胞構造内に存在し，内部に存在するHCV RNAやNSタンパク質は外部からのRNA分解酵素やプロテアーゼに対して保護されているものと考えられた．最近の知見では，HCV複製複合体は膜小胞構造内に保護されており，外部から投与したHCV RNAは複製複合体に到達できず，既に内部に取り込まれているHCV RNAがテンプレートとなってマイナス鎖RNAが合成され，それをもとに複数のプラス鎖RNAが合成される．複製に必要なNSタンパク質は継続的に供給される必要はなく，一度HCV複製複合体を形成し，RNA複製が開始されると継続的にRNAが産生されるものと推定できる．膜小胞内にはHCVゲノムの量に対してNSタンパク質の量は1000倍以上と大量に存在しているものの，実際複製に関わっているのはほんのわずかに過ぎず，NSタンパク質の大部分は膜小胞形成の役割を果たしているものと思われる．筆者らはスタチンの一つであるロバスタチンがHCV複製を阻害することを示したが，スタチンがゲラニルゲラニル化を抑制してウイルス複製を抑える可能性も指摘されている[13]．

4. 生体膜脂質のHCV粒子形成における役割

エンベロープウイルスは小胞体，ゴルジ体，形質膜などの細胞の生体膜を被って出芽するため，細胞の膜脂質はウイルス粒子形成に重要な役割を果たしているものと考えられる．さらに，ウイルス粒子の膜脂質が宿主細胞への感染過程に関与する例も報告されている．しかし，HCV粒子に含まれる脂質成分については解析が進んでおらず，その生理学的役割も不明であった．そこで筆者らは，培養細胞で産生させたHCV JFH–1粒子を，培養上清から，限外濾過，ショ糖密度勾配超遠心，ヘパリンアフィニティクロマトグラフィを組み合わせて，濃縮，粗精製し，このHCV粒子に含まれる脂質を生化学的に解析した．その結果，コレステロール／リン脂質モル比が細胞の膜分画に比べて有意に高値を示したことから，コレステロールに富んだ生体膜からの出芽，または粒子形成，分泌過程でのコレステロールとの会合の可能性が考えられた[4]．次にこのHCV粒子上の膜脂質がどのような役割を果たしているかを調べるため，HCV粒子表面をmethyl–B–cyclodextrin（B–CD）で処理してコレステロールを除去した後感染させたところ，B–CDの用量依存的に感染性が低下し，B–CD処理した粒子にコレステロールを添加したところその感染性は回復した[4]．また，コレステロールと親和性が高いスフィンゴ脂質の主要分子スフィンゴミエリンを加水分解するsphingomyelinase（SMase）でHCV粒子を処理することにより感染性の低下を観察した．これらのことはHCV genotype 1bのエンベロープを持つシュードタイプウイルスやキメラウイルスでも確認できた．以上から，ウイルス粒子表面のコレステロールとスフィンゴ脂質はウイルスの遺伝子型によらず感染に重要な役割を果たしていることが示された．次に，HCV粒子上のコレステロールが粒子の物性に与える影響を調べた．HCV産生細胞の培養上清をショ糖密度勾配遠心分画するとCore蛋白及びHCV RNAのピークは1.17 g/ml分画，感染性のピークは1.13 g/ml分画となる．このように，感染性のピークがウイルス遺伝子のそれに比べ低密度側に存在することは培養細胞系で作製したHCVの特徴の一つであるが，濃縮したこの培養上清をB–CD処理しコレステロール除去後に同様に遠心分画を行うと，

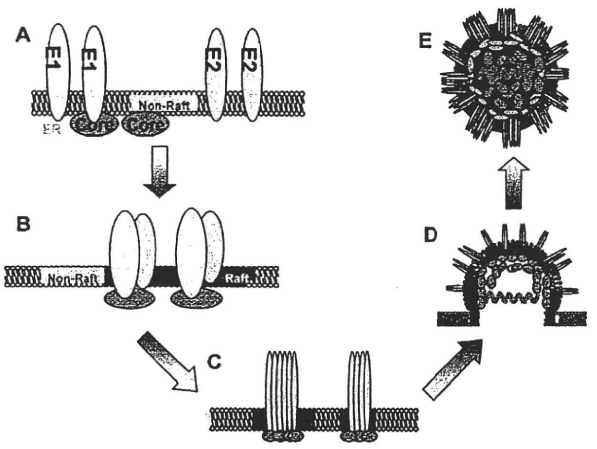

Fig. 3 Model of HCV assembly [14].

Core蛋白のピークは1.20 g/ml分画に移行し，感染性はいずれの分画も検出限界以下であった．さらに，B-CD処理後の培養上清にコレステロールを添加するとCore蛋白のピークは低密度側へシフトし感染性も回復した．このようなコレステロールの除去，および，その後の添加によるloss- and gain-of-functionは5 mg/ml B-CD処理で観察されるが，B-CD濃度を10 mg/mlへ上げた場合はコレステロール添加によって感染性の回復は見られない．これらのことから，HCV粒子表面のコレステロールは粒子構造の維持に役立っており，コレステロールを完全に除去してしまうと粒子構造は致命的なダメージを受ける，これに対し，部分的に除去した場合の構造変化は感染性を低下させるものの，その変化は再生可能なレベルである，と考えられた．次に，HCV粒子上のコレステロールまたスフィンゴ脂質が感染過程のどのステップに関与するのかを解析した．あらかじめコレステロール除去またはSMase処理を行ったHCV粒子の宿主細胞への吸着性は未処理ウイルスと同等であったのに対し，吸着後の細胞内への取り込みは，これらの前処理を施したHCVで顕著な低下が認められた．レセプター蛋白分子とともに標的細胞内へウイルスが侵入する過程に粒子コレステロール，スフィンゴ脂質が関与する可能性が示された．

前述のように，HCVゲノムは脂質ラフトの特徴である界面活性剤不溶性の膜分画で複製することが示され[6, 9]，HCV genotype 1のゲノム複製細胞またHCVが増殖するヒト肝細胞キメラマウスに脂質ラフト構成成分であるスフィンゴミエリンの合成阻害剤myriocin/ISP-1を添加，投与することによって，HCV複製効率は顕著に低下することが報告されている[10, 15]．このmyriocin/ISP-1またはセラミド輸送阻害剤HPA-12をHCV N株（genotype 1b）またJFH-1株のサブゲノムレプリコン細胞に加えることによって，N株ではHCVゲノム複製は阻害されるものの，JFH-1株では予想に反して複製の低下はほとんど認められなかった．しかしながら，興味深いことに，JFH-1のウイルス産生系では両薬剤の用量依存的にHCV産生は抑制された[14]．スフィンゴ脂質合成阻害剤の抗HCV効果の作用機序としてHCVゲノム複製阻害だけでなく粒子形成あるいは感染過程へも介入しうることが示唆された．

Fig. 3に脂質ラフトを利用したHCV粒子形成モデルを示す．HCV構造蛋白はERで合成され，E1およびE2蛋白はその3末端で膜にアンカーしている（A）．HCV構造蛋白はそれぞれ生体膜のうち脂質ラフトと結合する．さらに，コア蛋白とE1蛋白，E1とE2蛋白はそれぞれ結合する（B）．一度結合した脂質ラフト同士はコア蛋白同士の結合する力で安定化し，島状に次第に大きくなり，その過程でウイルス粒子構成の蛋白を集積させる（C）．さらに，膜上のコア蛋白同士が結合するエネルギーにより，膜は小胞を形成するようになる（D）．ここにコア蛋白と結合するHCV RNAが取り込まれることにより，HCV粒子を形成すると考えられる（E）．ウイルス粒子のコレステロールやスフィンゴ脂質を除くと感染性がなくなり，そこにコレステロールを加えると感染性が復活することから，ウイルス粒子膜上の脂質ラフトはウイルス粒子の感染性にも重要な役割を果たしているものと思われる．最近，HCV粒子形成に細胞内脂肪滴が重要な役割を果たすという発見がなされた[16]．脂肪滴に近接した生体膜でHCV粒子が形成されている可能性が考えられている．

5. おわりに

日本膜学会第31年会の生体膜関連シンポジウムのタイトルは「脂質低下療法時代の生体膜研究」ということであったので，最後に脂質低下療法によるHCV治療の可能性について考察してみたい．これまでの研究から，(i) ウイルス粒子膜は脂質に富んでおり感染に重要[14]，(ii) 細胞の形質膜の脂質も感染に重要[4]，(iii) 細胞の生体膜脂質はウイルスゲノム複製に重要[4]，(iv) 脂肪滴周辺の膜構造が粒子形成に重要[16]，などHCVはその生活環の多くのステップに脂質を必要としていることがわかってきた．そこで，筆者らはスタチン製剤でウイルス増殖を抑えることが可能かどうか調べた．ロバスタチンをHCV持続感染細胞に投与したところウイルス粒子産生量が強く抑制された．スタチン製剤は既に臨床の現場で広く使われており，安全性が確立している薬剤であり，

既にC型肝炎患者の治療も試みられている．スタチン製剤単独療法ではHCV治療に有効と無効の報告があり，意見が割れるところであるが，IFNとの併用の場合にはHCV治療に有効[17]という報告がある．

HCVはゲノム配列が多様で，大変変異しやすいウイルスである．そのエンベロープのアミノ酸配列を変えて宿主の免疫系から逃れ慢性持続感染を起こしていると考えられているだけでなく，IFNやリバビリンといった薬剤に対しても耐性を持つウイルスが出現しやすいことが知られている．新たな抗HCV薬として，ウイルスプロテアーゼやポリメラーゼといったウイルス複製に関与する酵素を標的とした薬剤の開発研究が盛んに行われているが，HIVと同様にこれらの薬剤についてもHCVは耐性変異を獲得することが報告されている．上記で報告した宿主のコレステロール産生系やスフィンゴ脂質の産生系をターゲットとし，感染した細胞側の働きを抑えてウイルス増殖を抑制する抗HCV薬の開発は耐性ウイルスが出現しにくい薬剤につながる期待がある．

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SYNCRIP (synaptotagmin-binding, cytoplasmic RNA-interacting protein) is a host factor involved in hepatitis C virus RNA replication

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ABSTRACT

Hepatitis C virus (HCV) RNA replication requires viral nonstructural proteins as well as cellular factors. Recently, a cellular protein, synaptotagmin-binding, cytoplasmic RNA-interacting protein (SYNCRIP), also known as NSAP1, was found to bind HCV RNA and enhance HCV IRES-dependent translation. We investigate whether this protein is also involved in the HCV RNA replication. We found that SYNCRIP was associated with detergent-resistant membrane fractions and colocalized with newly-synthesized HCV RNA. Knock-down of SYNCRIP by siRNA significantly decreased the amount of HCV RNA in the cells containing a subgenomic replicon or a full-length viral RNA. Lastly, an *in vitro* replication assay after immunodepletion of SYNCRIP showed that SYNCRIP was directly involved in HCV RNA replication. These findings indicate that SYNCRIP has dual functions, participating in both RNA replication and translation in HCV life cycle.

Introduction

Hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. HCV is an enveloped RNA virus with a positive-stranded RNA of 9.7 kb in length (Reed and Rice, 2000). It encodes a large polyprotein, which is then processed into structural proteins (C, E1, and E2) and nonstructural (NS) proteins, the latter of which participate in viral replication.

Besides the viral NS proteins, several host factors, including the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein (hVAP-33) (Gao et al., 2004; Hamamoto et al., 2005), polypyrimidine-tract-binding protein (PTB) (Aizaki et al., 2006; Chang and Luo, 2006; Domitrovich et al., 2005), La antigen (Domitrovich et al., 2005) and host geranylgeranylated proteins and fatty acids (Kapadia and Chisari, 2005) have been shown to be involved in some steps of HCV replication cycle. Some of these host factors, such as PTB and La autoantigen, were initially found to regulate HCV protein translations (Ali and Siddiqui, 1997; Ito and Lai, 1999) by virtue of their binding to the 5′ and 3′-untranslated regions (UTR) of HCV RNA. Later studies showed that some of these host factors also directly regulate HCV RNA replication either by participating in the formation of the RNA replication complex (e.g., VAP-33) (Gao et al., 2004) or by binding to the viral RNA (e.g., La, PTB) (Ali and Siddiqui, 1995; Chang and Luo, 2006). A recent study showed that another host protein, synaptotagmin-binding, cytoplasmic RNA-interacting protein (SYNCRIP), also named NS-1-associated protein (NSAP1), binds to the N-terminal of the core protein-coding region of HCV RNA and enhances HCV Internal Ribosomal Entry Site (IRES)-dependent translation (Kim et al., 2004).

SYNCRIP is a member of cellular heterogeneous nuclear ribonucleoprotein (hnRNP) family, to which PTB also belongs. hnRNPs are well-known for their abilities to bind to cellular proteins and RNAs to facilitate many biological processes. Interestingly, SYNCRIP has previously been shown to be involved not only in cellular processes but also in mouse hepatitis virus (MHV) RNA replication (Choi et al., 2004b). Since SYNCRIP binds to HCV RNA at a site close to the 5′-end of the RNA, it is likely that SYNCRIP may also affect the RNA replication of HCV. If this is the case, SYNCRIP will have duel functions in both RNA replication and protein translation, similar to other duel-purpose hnRNPs, such as PTB. Our goal in this study is to investigate whether SYNCRIP is involved in HCV RNA replication in addition to its role in translation.

Results

SYNCRIP relocalized to detergent-resistant membrane fraction in HCV replicon cells

It has been shown that HCV RNA replication occurs in detergent-resistant membrane (DRM) fractions (Ali et al., 2002; El-Hage and Luo,

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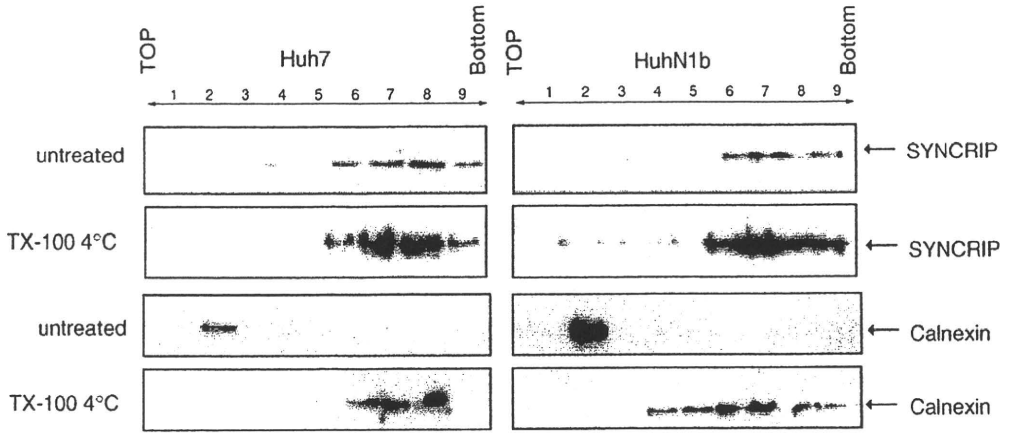

Fig. 1. Membrane flotation assay showed relocalization of SYNCRIP to DRM in HCV replicon cells. Cell lysates were prepared from HCV replicon (HuhN1b) or Huh7 cells by passing through a 25-gauge needle 20 times. Nuclei and unbroken cells were removed by centrifugation at 1000 ×g for 5 min in microcentrifuge at 4 °C. The supernatants treated with or without 1% TX-100 at 4 °C for 30 min were fractionated by discontinuous sucrose gradient centrifugation. Fractions were collected from the top, numbered from 1 to 9. Each fraction was concentrated by Centricon YM-30 (Millipore, MA) and immunoblotted by rabbit anti-SYNCRIP antibody or mouse anti-Calnexin antibody, respectively. SYNCRIP was found in both membrane and soluble fractions in the untreated Huh7 and HuhN1b cells, whereas in the HuhN1b cells, some SYNCRIP was localized to the DRM fractions. This phenomenon is not seen in the Calnexin profile.

2003; Mizutani et al., 2000). The nonstructural proteins of HCV are associated with the DRM structures containing Caveolin-2, strongly suggesting that the viral replication complex has properties of lipid rafts (Gao et al., 2004; Mizutani et al., 2000). To determine whether SYNCRIP is in the RNA replication complex, we performed membrane flotation analysis of HCV replicon cells, followed by immunoblotting with anti-SYNCRIP antibody to examine the possible presence of SYNCRIP in the detergent-resistant membrane fractions, where the HCV replication complexes reside. We found that SYNCRIP was present mostly in the cytosolic fractions (fractions 6–9, Fig. 1) in both Huh7 and HuhN1b cells, but a small fraction was associated with the membrane (fractions 2–4, Fig. 1). After treatment with Triton X-100 at 4 °C, some SYNCRIP was still associated with the membrane in HuhN1b cells; in contrast, almost all of SYNCRIP was solubilized in Huh7 cells. Longer exposure of immunoblotting was performed, and SYNCRIP was still not found in the DRM fractions of TX-100 treated Huh7 cells. As a control,

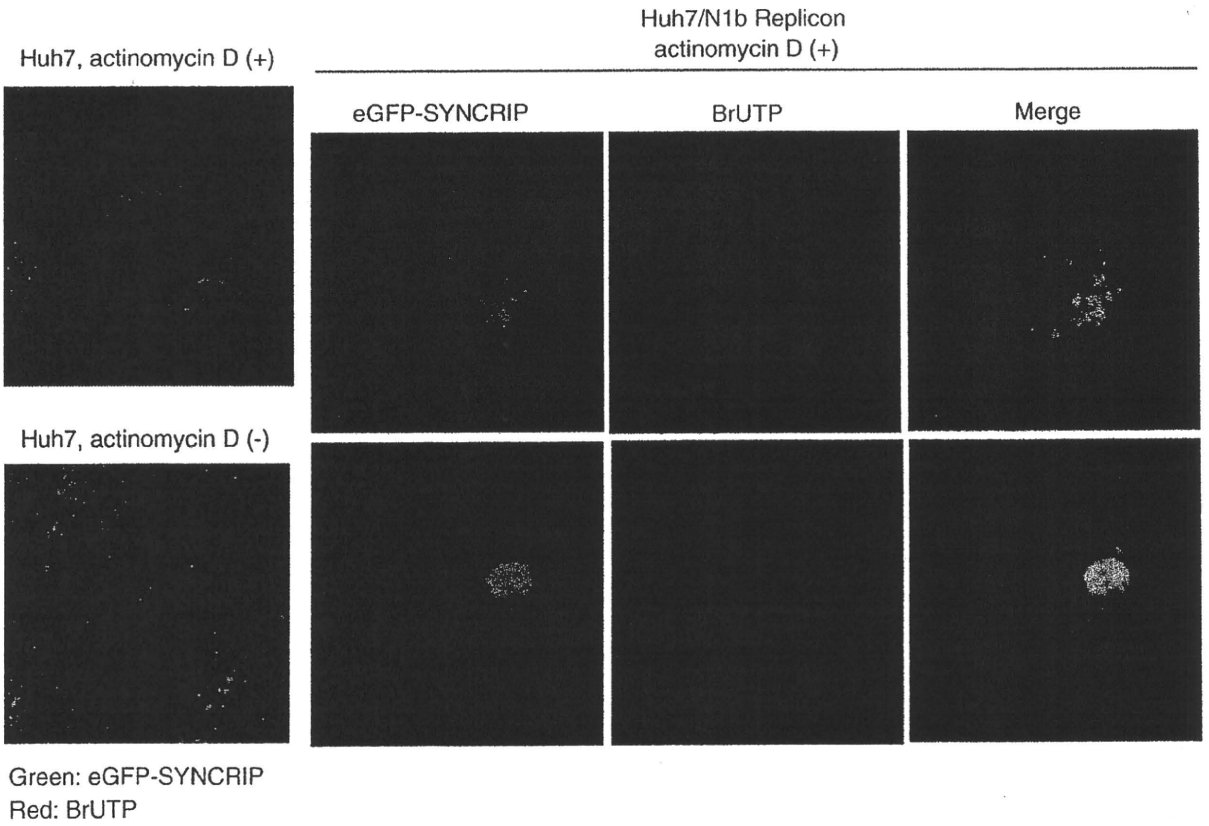

Fig. 2. SYNCRIP colocalization with de novo-synthesized HCV RNA in a HCV replicon cell. peGFP-SYNCRIP was transfected into Huh7 or HCV replicon (HuhN1b) cells by Fugene 6. Two days after transfection, Huh7 or HCV replicon cells were labeled with BrUTP for 15 min after one-hour treatment with Actinomycin D. Actinomycin D treatment inhibited BrUTP incorporation in Huh7 cells (left two panels), but not in HuhN1b replicon cells, where BrU label was detected in the cytoplasm (right 6 panels). Immunofluorescence staining was performed with sheep polyclonal antibody against BrdU (anti-BrdU) followed by Rhodamine-conjugated anti-sheep antibody (Jackson ImmunoResearch). Two different HuhN1b cells are shown, representing two different distribution patterns of BrUTP, as shown previously (Mizutani et al., 2000).

Calnexin, a marker protein of ER membrane, was concentrated exclusively in the membrane fractions (fraction 2–3) in the absence of detergent treatment in both Huh7 and HuhN1b cells (Fig. 1, panel 3). After the cells were treated with cold detergent, Calnexin was redistributed entirely to the soluble fractions, indicating that Calnexin was associated with detergent-soluble membrane, which is a known characteristic of unmodified ER. These data suggested that SYNCRIP is predominantly a cytoplasmic protein, but is relocalized to the DRM fractions (fraction 2–3 after TX-100 treatment) in the HuhN1b replicon cells. The relocalization of SYNCRIP protein, but not Calnexin, to the DRM fraction in the HuhN1b replicon cell indicated that SYNCRIP may be specifically recruited by HCV RNA to the replication complexes, since SYNCRIP binds to HCV RNA (Kim et al., 2004).

SYNCRIP colocalized with de novo synthesized RNA in HCV replicon cells

Previous studies on HCV replicon cells have shown that the newly synthesized HCV RNA and the viral nonstructural proteins colocalized with each other on the distinct speckle-like structure in the cytoplasm of the replicon cells (Gosert et al., 2003; Mizutani et al., 2000). To examine whether SYNCRIP is associated with HCV RNA synthesis in the speckle structures, BrUTP labeling was performed in HCV replicon cells transfected with peGFP-SYNCRIP (a gift from Dr. Mizutani, The University of Tokyo). Briefly, 2 days after transfection of peGFP-SYNCRIP, BrUTP was transfected into actinomycin D-pretreated cells (Kanestrom et al., 1998). Immunofluorescence staining with sheep anti-BrdU polyclonal antibody (Biodesign, ME) was then performed (Kanestrom et al., 1998). Under this condition, all of the BrU-label represents HCV RNA since the cellular transcription is inhibited by actinomycin D treatment. The BrU-labeled RNA was present either in distinct speckle-like structures or in large spherical particles in the cytoplasm of the replicon cell (Fig. 2), consistent with our previous report (Mizutani et al., 2000). These two patterns probably represent two different states of viral RNA synthesis. No BrU-labeled RNA was found in Huh7 cells without an HCV replicon. SYNCRIP was also localized in the cytoplasm in Huh7 cells without HCV replicon, but in a

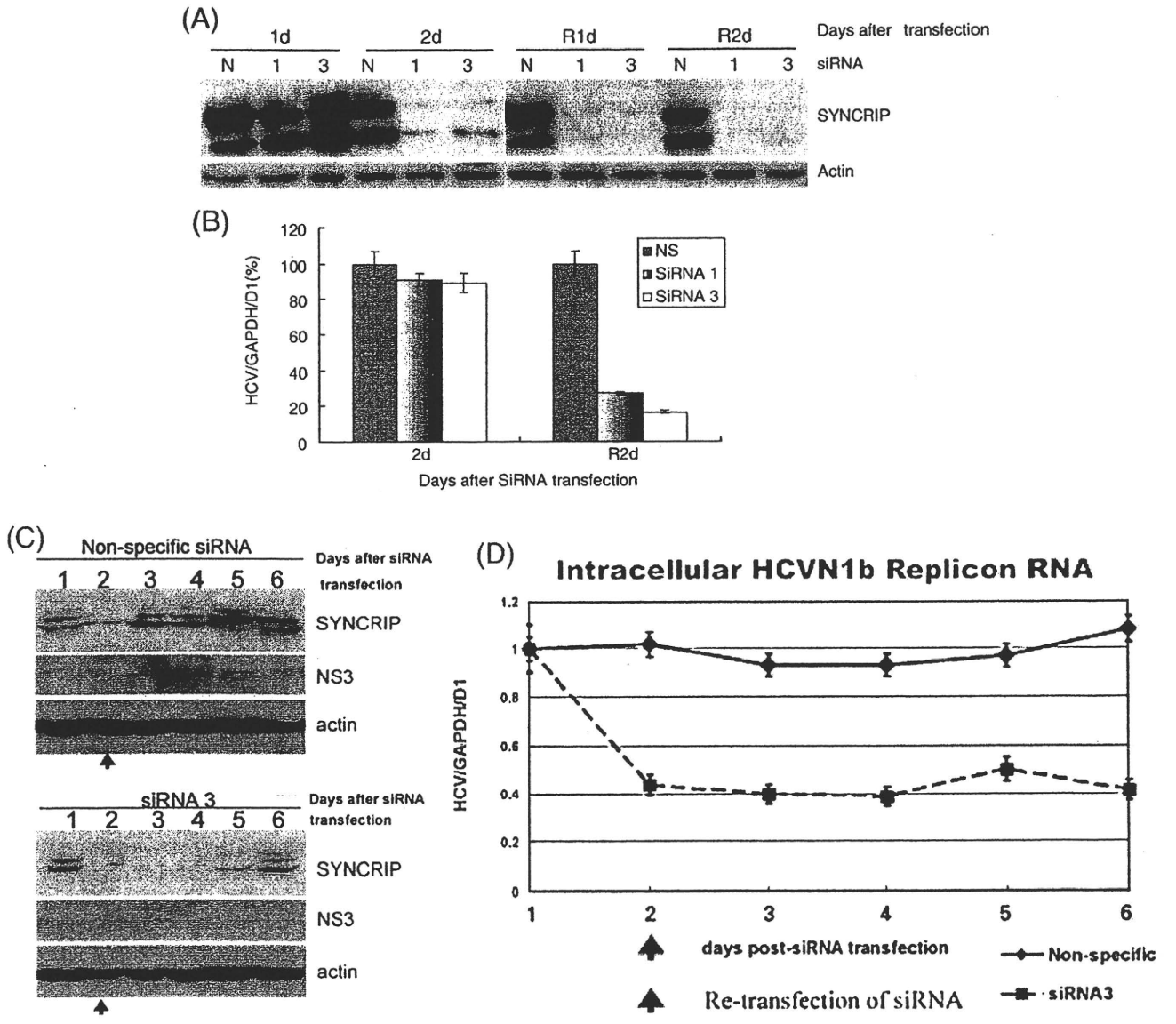

Fig. 3. SYNCRIP knock-down by siRNA in HCV replicon cells. The siRNAs against SYNCRIP (siRNA 1 and 3) (Choi et al., 2004b) or nonspecific siRNA were transfected into HCV replicon cells. Two days after the first transfection, each siRNA was re-transfected into the same cells to ensure complete knock-down of SYNCRIP. Endogenous SYNCRIP protein levels were monitored by immunoblotting (A), and HCV RNA levels were detected by realtime RT-PCR (B). (C), immunoblotting of SYNCRIP and NS3 expression in SYNCRIP siRNA knock-down replicon cells. (D), intracellular replicon RNA level was examined by realtime RT-PCR. R1d and R2d, one or 2 days after re-transfection of siRNA. siRNA 1 and 3, two different clones of siRNA; N, nonspecific siRNA.

more diffuse pattern than that of BrU label in HCV replicon cells. It was found that eGFP-SYNCRIP was partially colocalized with BrU-labeled RNA in the replicon cells (Fig. 2), indicating that only a portion of SYNCRIP was recruited to the HCV RNA replication site. This finding is consistent with the fractionation profile, which showed that SYNCRIP is primarily a cytosolic protein and that only a portion of SYNCRIP is relocalized to the DRM fractions in the replicon cells (Fig. 1). This phenomenon was also observed with PTB in the replicon cell (Aizaki et al., 2006), in which only a small portion of PTB was relocalized to the cytoplasm, whereas the majority remained in the nucleus. These results suggested that a portion of SYNCRIP is localized to the HCV replication complex, implying that SYNCRIP is involved in HCV RNA replication.

In vivo knock-down of SYNCRIP suppressed HCV replication

To determine the biological role of SYNCRIP in HCV RNA replication, we monitored HCV RNA levels in HCV replicon cells in which the endogenous SYNCRIP was knocked down with the RNA interference method (Aizaki et al., 2006; Wagner and Garcia-Blanco, 2002). HuhHyg replicon cells were transfected with either SYNCRIP-specific (siRNA 1 and 3) (Choi et al., 2004b) or nonspecific (NS) siRNA. Protein analysis by immunoblotting was performed with rabbit polyclonal anti-SYNCRIP antibody, and HCV RNA level was monitored by using Taqman quantitative realtime RT-PCR (Gao et al., 2004). The cells transfected with SYNCRIP siRNA showed a significant reduction of the endogenous SYNCRIP by day 2 post-transfection (Fig. 3A). One day after re-transfection with the same siRNAs respectively, almost no endogenous SYNCRIP could be detected (R1d, Fig. 3A). Correspondingly, SYNCRIP siRNA-transfected HCV replicon cells showed a 70–80% reduction of HCV RNA by day 2 after re-transfection with the siRNA as compared to that in the cells transfected with the nonspecific siRNA (R2d, Fig. 3B). The lag time of more than 1 day between the drop of SYNCRIP and that of HCV RNA was probably due to the relative stability of the HCV RNA.

HuhN1b replicon cells were also used to confirm the result obtained using HuhHyg replicon cells. SYNCRIP knock-down was achieved by siRNA transfection, and the viral protein and RNA levels were examined in a time-course study. While the nonspecific siRNA did not significantly affect SYNCRIP expression, the expression levels of SYNCRIP were dramatically decreased after the transfection of specific siRNA against SYNCRIP (siRNA 3) (Fig. 3C). Correspondingly, a 50% decrease in intracellular replicon RNA were detected from day 2 p. t. (Fig. 3D). The decrease in NS3 expression was also detected in siRNA 3-transfected cells; however, the viral protein was not decreased until 4 days after siRNA transfection (Fig. 3C). These results suggest that SYNCRIP affects both HCV RNA translation and RNA replication, but exerts these effects through different mechanisms.

It has been reported that SYNCRIP interacts with HCV RNA fragment spanning nt 342 to 374, corresponding to the N-terminus of the core protein-coding region (Kim et al., 2004). Since this region is immediately downstream to the neomycin-phosphotransferase gene in the HCV replicon, it is possible that the observed involvement of SYNCRIP in the HuhN1b replicon cells was due to the possible effects of SYNCRIP on the expression of phosphotransferase. To rule out this possibility, we further examined the role of SYNCRIP in the replication of HCV full-length RNA (pHCV-1b-hyb) without the neomycin phosphotransferase gene. Huh7 cells were first transfected and re-transfected with SYNCRIP-specific siRNA to knock-down the endogenous SYNCRIP protein; 1 day after re-transfection of siRNA, the cells were transfected with the replication-competent full-length HCV RNA (HCV-1b). The SYNCRIP expressions and NS3 levels in siRNA-transfected cells were examined by immunoblotting. SYNCRIP expression was significantly decreased in cells transfected with specific siRNA (siRNA 1 or 3) (Fig. 4A). Correspondingly, NS3 expression was also affected by the specific SYNCRIP siRNA; in siRNA 1- and siRNA 3-transfected cells, NS3 was detected in the first 2 days post-transfection of HCV full-length RNA, but became undetectable thereafter, whereas in the nonspecific siRNA-transfected cells, NS3 was detected up to 4 days after HCV RNA transfection (Fig. 4A). Total intracellular HCV RNA was determined at various days by quantitative realtime RT-PCR. In the cells transfected with the nonspecific siRNA, HCV RNA titer gradually increased during the first 72 h post-transfection, in agreement with the published report (Choi et al., 2004a) (Fig. 4B). In contrast, in the cells transfected with the SYNCRIP-specific siRNA, HCV RNA titer decreased steadily over the same period of time (Fig. 4B). Although NS3 was detected in the first 2 days after HCV RNA transfection in SYNCRIP-knocked down cells (Fig. 4A), there was no sign of HCV replication (Fig. 4B). The intracellular HCV RNA level normalized by NS3 expression level was shown in Fig. 4C. Regardless the NS3 expression detected in SYNCRIP knock-down cells, HCV RNA titer constantly decreased after full-length HCV RNA transfection. There was a slight increase in intracellular HCV RNA level at day 5 post-transfection of HCV full-length RNA, probably due to the increase in SYNCRIP protein level. This result suggested that endogenous SYNCRIP is directly involved in HCV replication, but not through the suppression of the expression of neomycin phosphotransferase gene. These results combined indicate that SYNCRIP is involved in HCV replication by affecting either HCV RNA replication or translation, or both.

SYNCRIP inhibited HCV RNA replication in vitro

The siRNA knock-down approaches showed that once SYNCRIP protein level was decreased, the HCV RNA titer would be correspondingly decreased. Since SYNCRIP has been shown to be directly involved in HCV translation (Kim et al., 2004), the inhibition of HCV RNA replication in SYNCRIP-knock-down cells may have resulted from the indirect effect of inhibition of translation; namely, the viral NS protein synthesis was inhibited, and thereby viral RNA synthesis was decreased.

To distinguish the effect of SYNCRIP on RNA replication from that on translation, we designed experiments to separate viral RNA replication from viral translation. We employed an *in vitro* replication assay using crude membrane fractions of the HCV subgenomic replicon cells (Ali et al., 2002; Gao et al., 2004), after the endogenous SYNCRIP had been knocked down by the siRNA approach. We also performed *in vitro* RNA replication assay after SYNCRIP was depleted with the anti-SYNCRIP antibody from the cell lysates.

Immunoblotting showed that the amount of SYNCRIP in the HuhN1b replicon cells was substantially reduced by the specific siRNA treatment for 2 days (Fig. 5A). At this time, the amount of NS5A was only partially reduced. Cell lysates from siRNA-transfected replicon cells were treated with TX-100 at 4 °C for 30 min and fractionated by sucrose gradient centrifugation to isolate DRM fraction (Aizaki et al., 2006; Ali et al., 2002). The DRM fractions from these cell lysates were then used for *in vitro* replication assay. The HCV RNA synthesis was detected as single band of ^{32}P-labeled RNA. The result showed that there was no detectable RNA replication activity at all in the DRM fractions from the SYNCRIP siRNA-transfected replicon cells when compared with those from the non-transfected or nonspecific siRNA-transfected replicon cells (Fig. 5A). Since there was still a significant amount of NS5A remaining in the siRNA-transfected cells, the total lack of the *in vitro* replication activity in SYNCRIP knocked-down replicon cells suggested a direct role of SYNCRIP in HCV RNA replication.

We further performed an immunodepletion experiment to remove SYNCRIP form the DRM fraction and assessed the effects on HCV RNA replication *in vitro*. For immunodepletion, the DRM fractions from replicon cell lysates were incubated with a rabbit anti-SYNCRIP polyclonal antibody to deplete the endogenous SYNCRIP from the lysate. After incubation, samples were used for cell-free synthesis of

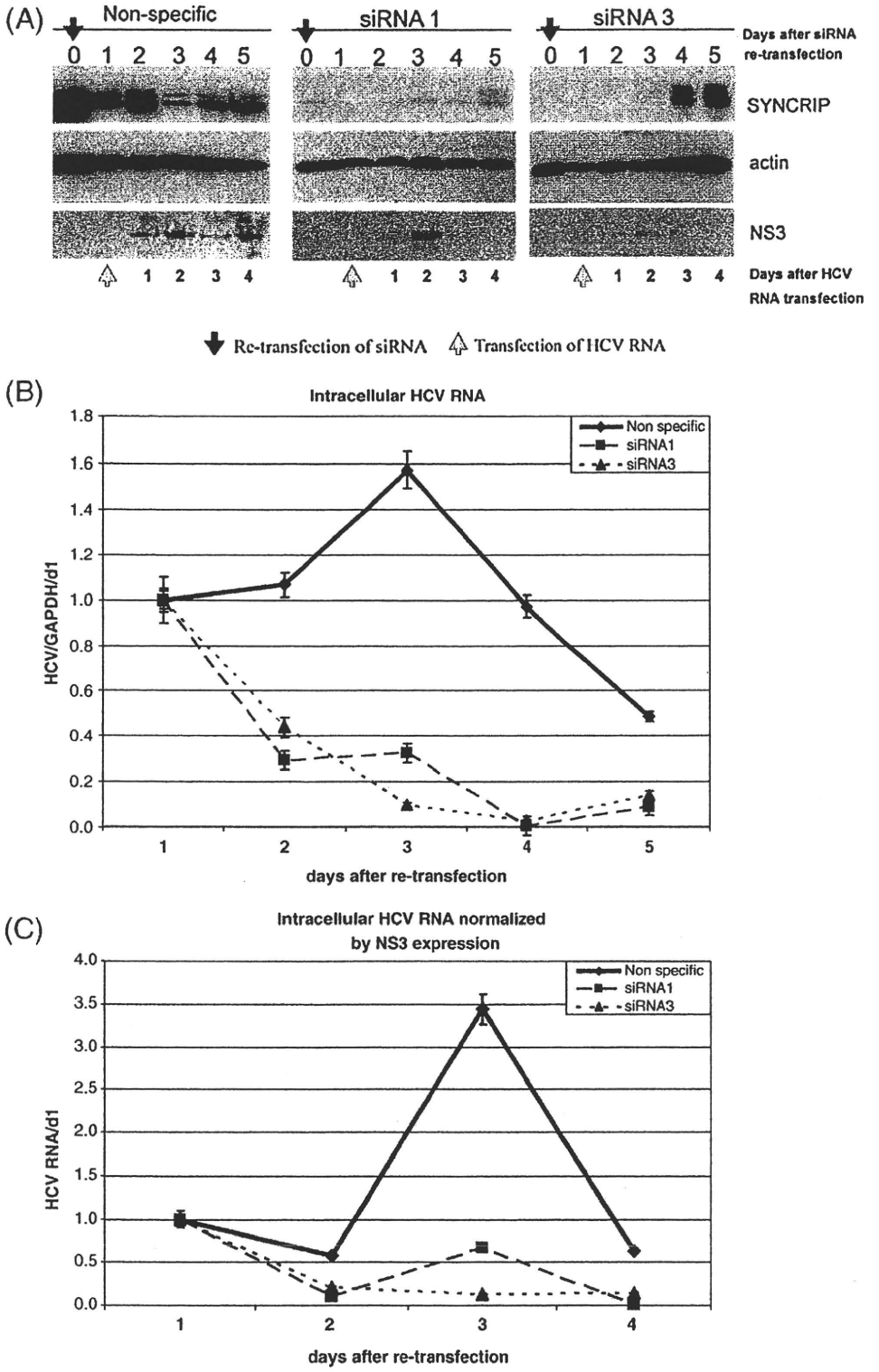

Fig. 4. Deficiency of full-length HCV replication in SYNCRIP knock-down Huh7 cells. The siRNAs against SYNCRIP (siRNA 1 and 3) or nonspecific siRNA were transfected into Huh7 cells twice to knock-down endogenous SYNCRIP level as described in Fig. 3. One day after re-transfection of siRNA, *in vitro* synthesized full-length HCV-1b-hyb RNA was transfected to siRNA-transfected Huh7 cells respectively. (A), immunoblotting of endogenous SYNCRIP after siRNA re-transfection, and NS3 expression levels at various days after HCV RNA transfection. (B), intracellular HCV RNA levels determined by quantitative RT-PCR, and (C), intracellular HCV RNA levels normalized by NS3 expression levels. Huh7 cells were transfected with SYNCRIP-specific or nonspecific siRNA as in (A). One day after siRNA transfection, the replication-competent full-length HCV RNA was transfected into the cells, and HCV RNA levels were detected by realtime RT-PCR on different days after the HCV RNA transfection. The relative amounts of HCV RNA are expressed as in Fig. 3B.

HCV RNA. The results showed that the treatment with anti-SYNCRIP antibody inhibited the replication activity in an antibody concentration-dependent manner, whereas a control anti-Ig antibody did not inhibit any activity at the same or an even higher antibody concentration (Fig. 5B and data not shown). As a control, anti-VAP33 (VAP-A) antibody also inhibited HCV RNA replication, similar to the previous result (Hamamoto et al., 2005), whereas anti-Calnexin antibody did not.

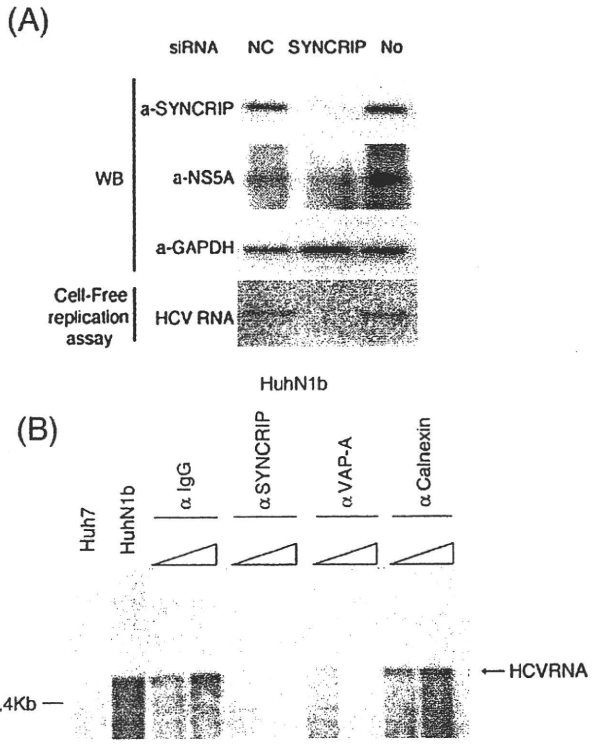

Fig. 5. siRNA-knock-down or immunodepletion of SYNCRIP inhibited HCV replication activity in *in vitro* replication assay. (A) SYNCRIP was knocked down by transfection two times with specific siRNA in HCV replicon cells as in Fig. 3. SYNCRIP, NS5A and GAPDH protein expression were determined by immunoblotting. Cell-free RNA replication assays using the DRM fraction isolated from the cell lysates, as described in Material and methods, were performed. ^{32}P-CTP-labeled HCV RNA product was detected by autoradiography after separation by agarose gel electrophoresis. NC, nonspecific siRNA control; SYNCRIP, SYNCRIP-specific siRNA; No, no siRNA transfection. (B) Partially purified lysates of HCV replicon cells were incubated with anti-IgG, anti-SYNCRIP, anti-VAP-A (VAP-33), or anti-Calnexin antibodies. Then samples were incubated with α-^{32}P-CTP in a cell-free RNA-dependent RNA polymerase assay. The RNA product was separated by formaldehyde agarose gels and identified by autoradiography.

These results combined suggested that SYNCRIP is directly involved in HCV RNA replication, in addition to its role in regulating the translation of HCV RNA. Since SYNCRIP is colocalized with the newly synthesized HCV RNA, it is likely that SYNCRIP is a part of the HCV RNA replication complex and participates in viral RNA synthesis.

Discussion

Our results show that SYNCRIP can modulate HCV RNA replication. It has been previously reported that SYNCRIP can also enhance HCV IRES-dependent translation (Kim et al., 2004). Thus, similar to PTB and La autoantigen, SYNCRIP has dual functions in HCV life cycle. This may be a common characteristic of HCV RNA-binding proteins.

In our study, the relocalization of SYNCRIP to the DRM fractions in HCV replicon cells indicates that SYNCRIP is associated with the RNA replication complex, which is localized in this membrane fraction. It is interesting to note that the distribution of Calnexin was slightly different between the control and the replicon cells; there was some shift of Calnexin toward the lighter sucrose gradient fractions, probably cased by the alteration of cellular membrane structures and the associations of HCV NS proteins to ER membrane structure in HCV replicon cells (Egger et al., 2000; El-Hage and Luo, 2003; Gosert et al., 2003; Mottola et al., 2002). Nevertheless, SYNCRIP was clearly localized in the DRM fraction, whereas Calnexin was not.

The immunodepletion experiments in the current and previous studies have shown that antibody against PTB, hVAP-A, hVAP-B, and SYNCRIP can inhibit HCV RNA replication activities specifically. Previous studies have suggested that the HCV RNA replication complexes are protein complexes with the newly-synthesized RNA being contained within (Yang et al., 2004), and that subtilisin protease treatment could disrupt the replication complexes. However, it was also reported that the HCV replication complexes were resistant to proteinase K treatment at room temperature (Aizaki et al., 2004; Quinkert et al., 2005). The latter study suggested that the HCV replication complexes were very compact, and therefore the accessibility of immunoglobulin to the specific protein target in the replication complex may be limited. However, the ability of these antibodies to inhibit HCV RNA replication suggested that those complexes may not be so compact and are accessible by immunoglobulin molecules under these conditions.

The genome of positive-stranded RNA viruses, such as HCV, poliovirus, and coronavirus, serve as a template for both translation and the synthesis of negative-strand RNA, the latter of which is, in turn, the template for synthesizing more positive-strand RNA. The positive-strand RNA can also be packaged to form new viral particles. Since the same positive-strand RNA can participate in different steps of the viral life cycle, the temporal or spatial regulation is very important. It is likely that the regulation is through RNA–protein interactions. When in complex with specific RNPs, the RNA can be utilized specifically in different steps. With limited numbers of genes in the viral genome, the regulation likely requires the participation of various host factors interacting with the viral RNA or viral proteins.

There are many known host and viral RNA-binding proteins that can facilitate positive-strand RNA replication, such as Tat protein binding to the TAR structure in HIV1 RNA (Dingwall et al., 1989; Wagner and Garcia-Blanco, 2002) and poly(rC)-binding protein binding to the cloverleaf structure of poliovirus RNA (Blyn et al., 1996; Gamarnik and Andino, 2000). However, not so many RNA-binding proteins have been reported to have dual functions in viral RNA replication and translation. Recently, La and PTB, are found to regulate both RNA replication and translation of HCV, probably as a result of their ability to bind to HCV RNA (Aizaki et al., 2006; Domitrovich et al., 2005). Host factors with dual-regulatory functions may play important roles in switching the RNA from translation to replication or replication to translation. For example, PCBP regulates translation–replication switch in poliovirus life cycle (Back et al., 2002; Gamarnik and Andino, 1998). It was reported previously that stem–loop I and II are critical for HCV RNA replication, and stem–loop II, III, and IV are important for HCV RNA translation (El-Hage and Luo, 2003; Fukushi et al., 2001; Qi et al., 2003). La autoantigen was shown to bind to loop IV of HCV 5′NTR (Ali and Siddiqui, 1997). Although there is no evidence that La binds to stem–loop I or II, La can, nevertheless, regulate HCV replication (Domitrovich et al., 2005). Similarly, SYNCRIP was reported to bind at nt 342 to 374 (Kim et al., 2004), a region essential for HCV IRES-driven translation but not HCV replication. Yet we found significant positive regulatory effect of SYNCRIP in HCV RNA replication. It is possible that the binding of SYNCRIP to HCV RNA alters the secondary structure of the RNA or recruits other required factors to facilitate the assembly of the replication complex.

The mechanism of switching between translation and replication of HCV RNA is still unclear; conceivably, it may be regulated by these dual-function proteins which are involved in both replication and translation. It will be interesting to determine in the future whether the relative ratio of these proteins may trigger the switch.

Materials and methods

Cells

Huh7 cells were grown at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids. Huh7N1b and HuhHyg replicon cells

harboring an HCV subgenomic replicon RNA derived from the HCV-N strain (Guo et al., 2001) were grown in the same medium containing 0.5 mg/ml of G418 or 100 μg/ml of Hygromycin (Mizutani et al., 2000).

Antibodies and drugs

The primary antibodies used for the analyses in this study were sheep anti-BrdU polyclonal antibody (BioDesign, ME), mouse anti-BrdU monoclonal antibody (Caltag, CA), anti-Calnexin monoclonal antibody (Abcam, MA), anti-GS27 monoclonal antibody (Abcam, MA). Brefeldin A and Nocodazole were purchased from Sigma, and Actinomycin D was from Fisher. The polyclonal anti-SYNCRIP antibody was generated in rabbits by peptide (amino acid 140 to 152) injection (Mizutani et al., 2000).

Labeling and immunofluorescence staining of de novo-synthesized viral RNA

Labeling of de novo-synthesized viral RNA, immunofluorescence staining and confocal microscopy were modified from the previously described procedures (Kanestrom et al., 1998). Briefly, Huh7 or replicon cells were plated on 8-well chamber slides at a density of 1×10^4 cells per well. Two days after seeding, cells were incubated with actinomycin D (10 μg/ml) for 1 h to inhibit cellular RNA synthesis. Subsequently, 2 mM of bromouridine triphosphate (BrUTP) was transfected into cells at 4 °C for 15 min using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals, IN). The cells were washed with phosphate-buffered saline (PBS) twice and cultured at 37 °C for different incubation durations with DMEM supplemented with 10% FBS. After incubation, cells were washed twice with PBS and subsequently fixed by 4% formaldehyde for 1 h at 4 °C. For permeabilization, the cells were treated with 0.1% Triton X-100 (TX-100) (Sigma-Aldrich, St. Louis, MO) in PBS supplemented with 1% FBS for 30 min at room temperature. Primary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and incubated with cells for 1 h at room temperature. After three washes in PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated or Rhodamine-conjugated secondary antibodies diluted at a 1:100 with PBS containing 5% BSA for 1 h at room temperature. The cells were then washed three times in PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Membrane flotation, detergent solubilization assay

The membrane flotation assay was performed as previously described (Mizutani et al., 2000). Briefly, cells were first lysed in 1 ml of hypotonic buffer [10 mM Tris–HCV (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$] and passed through a 25-gauge needle 20 times. Nuclei and unbroken cells were removed by centrifugation at 1000 g for 5 min in microcentrifuge at 4 °C. Cell lysates were then mixed with 3 ml of 72% sucrose in low-salt buffer [LSB, comprising 50 mM Tris–HCl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$] and overlaid with 4 ml of 55% sucrose in LSB, followed by 1.5 ml of 10% sucrose in LSB. The sucrose gradient was centrifuged at 38,000 rpm in a Beckman SW41 Ti rotor for 14 h for 4 °C. After centrifugation, 1-ml fractions were taken from the top of the gradient, and each was added 1.7 ml of LSB to dilute sucrose and concentrated by being passed through a Centricon YM-30 filter unit (Millipore, Bedford, MA). One half of each sucrose gradient fraction was separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. After blocking, the membrane was incubated with the primary antibody for 1 h at 37 °C, followed by the appropriate species-specific horseradish peroxidase conjugate, for an additional 1 h at 37 °C. Bound antibody was detected by the ECL-plus system (Amersham, Piscataway, NJ).

Transfection of siRNAs and HCV full-length RNA

The siRNAs against SYCRIP are 19-nt sequences located at nt 189–107 and nt140–1438, respectively, of SYNCRIP open reading frame (ORF) and were synthesized by Integrated DNA Technologies, Inc. (Coralville, IW). siRNAs were designed to target two different sites of the human SYNCRIP gene (5′-CUAUCGUGGUGGAUAUGAAGATT-3′, and 5′-AGACAGUGAUCUCUCUCAUGUTT-3′) chosen with the siRNA target finder software from Ambion (http://www.ambion.com/techlib/misc/siRNA_finder.html) (Choi, Mizutani, and Lai, 2004b). Replicon or Huh7 cells were grown in 10% FBS-DMEM without antibiotics. For transfection, cells were plated to a density of 10^5 cells per well in a 24-well plate on day 1. Three microliters of a 20-μM stock of siRNA duplex was mixed with 47 μl of Opti-MEM (Invitrogen, CA) on day 2. In a separate tube, 3 μl of Lipofectamine 2000 (Invitrogen, CA) was resuspended in 12 μl of Opti-MEM, followed by incubation at RT for 7 min. The two mixtures were combined and allowed to sit at RT for 25 min. After the incubation, 35 μl of Opti-MEM was added and the 100 μl mixture was directly added to the well containing 500 μl of growth medium. On day 3, cells were trypsinized and split into a well of the 12-well plate. On day 4, cells were re-transfected using 6 μl of siRNA with 6 μl of Lipofectamine 2000. On day 5, cells were harvested either for Western blot analysis or for RNA isolation.

pHCV-1bhyb, which contains a full-length HCV RNA hybrid sequence of genotype 1a and 1b under the control of T7 polymerase promoter, has been described previously (Choi et al., 2004a). Full-length HCV RNA was *in vitro* transcribed through T7 promoter to obtain a positive-sense HCV RNA of about 9.6 kb. HCV full-length RNA was then transfected into cells 1 day after siRNA re-transfection with Mirus Trans-IT mRNA transfection reagents (Mirus Bio, WI). Briefly, 1 μl of Booster reagent and 1 μl of Trans-IT reagent were added into 100 μl OPTI-MEM sequentially, followed by 1.5 μg of *in vitro* transcribed HCV RNA. The mixture was incubated for 3 min at RT and added into each well of a 12-well plate containing 1 ml of fresh DMEM supplemented with 10% FBS. Cellular RNA was isolated from each well at 0 to 4 days after HCV RNA transfection.

Cell-free replication assay and immunodepletion experiment

Cell lysate of replicon or control Huh7 cells were prepared by a modified protocol (Ali, Tardif, and Siddiqui, 2002). The cells grown in 100-mm-diameter dishes were washed with cold washing buffer (150 mM sucrose, 30 mM HEPES [pH 7.4], 33 mM ammonium chloride, 7 mM KCl, 4.5 mM magnesium acetate), followed by treatment with lysolecithin buffer (250 μg/ml of washing buffer) for 2 min. Three milliliters of washing buffer were added to each culture plate. The buffer was removed by aspiration. The cells were collected by scraping in 120 μl of incomplete replication buffer (100 mM HEPES [pH 7.4]; 50 mM ammonium chloride; 7 mM potassium chloride; 1 mM spermidine; 1 mM [each] ATP, GTP, and UTP; 10 μM CTP), transferred to a new tube, and lysed gently by pipetting 15 times. The cell suspension was centrifuged at 1600 rpm in a microcentrifuge for 5 min at 4 °C.

For immunodepletion experiment, 40 μl of cytoplasmic fraction (supernatant) obtained as above was treated with 1% Nonidet P-40 (NP-40) (Boehringer Mannheim, Quebec, Canada) at 4 °C for 1 h and incubated with 0.1 μg or 1.0 μg of the indicated antibody with an adjusted amount of PBS at 4 °C for 4 h with rotation. After incubation, sample was incubated with ^{32}P-CTP (30 μCi; 800 Ci/mmol), 10 μg of actinomycin D per ml, and 800 U of RNase inhibitor per ml (Promega Corporation, Wis.) for 3 h at 30 °C. Extraction of RNA from the total mixture was performed with the TRI Regent (Molecular Research Center, Inc., Cincinnati, OH). The RNA were precipitated and eluted in 10 μl of RNase-free water. The replication products were analyzed by gel electrophoresis on 1% formaldehyde agarose gel.

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Involvement of Creatine Kinase B in Hepatitis C Virus Genome Replication through Interaction with the Viral NS4A Protein▽

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Persistent infection with hepatitis C virus (HCV) is a major cause of chronic liver diseases. The aim of this study was to identify host cell factor(s) participating in the HCV replication complex (RC) and to clarify the regulatory mechanisms of viral genome replication dependent on the host-derived factor(s) identified. By comparative proteome analysis of RC-rich membrane fractions and subsequent gene silencing mediated by RNA interference, we identified several candidates for RC components involved in HCV replication. We found that one of these candidates, creatine kinase B (CKB), a key ATP-generating enzyme that regulates ATP in subcellular compartments of nonmuscle cells, is important for efficient replication of the HCV genome and propagation of infectious virus. CKB interacts with HCV NS4A protein and forms a complex with NS3-4A, which possesses multiple enzyme activities. CKB upregulates both NS3-4A-mediated unwinding of RNA and DNA in vitro and replicase activity in permeabilized HCV replicating cells. Our results support a model in which recruitment of CKB to the HCV RC compartment, which has high and fluctuating energy demands, through its interaction with NS4A is important for efficient replication of the viral genome. The CKB-NS4A association is a potential target for the development of a new type of antiviral therapeutic strategy.

Hepatitis C virus (HCV) infection represents a significant global healthcare burden, and current estimates suggest that a minimum of 3% of the world's population is chronically infected (4, 19). The virus is responsible for many cases of severe chronic liver diseases, including cirrhosis and hepatocellular carcinoma (4, 16, 19). HCV is a positive-stranded RNA virus belonging to the family *Flaviviridae*. Its ~9.6-kb genome is translated into a single polypeptide of about 3,000 amino acids (aa), in which the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B reside in the C-terminal half region (6, 34, 44). NS4A, a small 7-kDa protein, functions as a cofactor for NS3 to enhance NS3 enzyme activities such as serine protease and helicase activities. The hydrophobic N-terminal region of NS4A, which is predicted to form a transmembrane α-helix, is responsible for membrane anchorage of the NS3-4A complex (8, 44, 50), and the central region of NS4A is important for the interaction with NS3 (10, 44). A recent study demonstrated the involvement of the C terminus of NS4A in the regulation of NS5A hyperphosphorylation and viral replication (28).

The development of HCV replicon technology several years ago accelerated research on viral RNA replication (7, 44). Furthermore, a robust cell culture system for propagation of infectious HCV particles was developed using a viral genome of HCV genotype 2a, JFH-1 strain, enabling us to study every process in the viral life cycle (27, 47, 54). RNA derived from genotype 1a, HCV H77, containing cell-culture adaptive mutations, also produces infectious viruses (52). Using these systems, it has been reported that the HCV genome replicates in a distinct, subcellular replication complex (RC) compartment, which includes NS3-5B and the viral RNA (2, 14, 33). The RC forms in a distinct compartment with high concentrations of viral and cellular components located on detergent-resistant membrane (DRM) structures, possibly a lipid-raft structure (2, 41), which may protect the RC from external proteases and nucleases. Almost all processes in viral replication are dependent on the host cell's machinery and involve intimate interaction between viral and host proteins. However, the functional roles of host factors interacting with the HCV RC in viral genome replication remain ambiguous.

To gain a better understanding of cellular factors that are components of the HCV RC and that function as regulators of viral replication, a comparative proteomic analysis of DRM fractions from HCV replicon and parental cells and subsequent RNA interference (RNAi) silencing of selected genes were performed. We identified creatine kinase B (CKB) as a key factor for the HCV genome replication. CKB catalyzes the reversible transfer of the phosphate group of phosphocreatine

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(pCr) to ADP to yield ATP and creatine and is known to play important roles in local delivery and cellular compartmentalization of ATP (48, 51). The findings obtained here suggest that recruitment of CKB to the HCV RC, through CKB interaction with NS4A, is essential for maintenance or enhancement of viral replicase activity.

MATERIALS AND METHODS

Cell lines, antibodies, and reagents. Human hepatoma cell line Huh-7.5.1 (54) was kindly provided by Francis V. Chisari. Cell lines carrying subgenomic replicon RNAs, namely, SGR-N (41) and SGR-JFH1 (23), were derived from the HCV-N (17) and JFH-1 strains (24), respectively. Mouse monoclonal antibodies (MAbs) against HCV NS3 (Chemicon, Temecula, CA), NS4A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), NS5A (Biodesign, Saco, ME), NS5B (2), FLAG (M2; Sigma-Aldrich, St. Louis, MO), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon), and Flotillin-1 (BD Biosciences, San Jose, CA) and polyclonal antibodies (PAbs) against CKB (mouse [Abnova, Taipei, Taiwan], goat [Santa Cruz]), hemagglutinin (HA; Sigma-Aldrich), and FLAG (Sigma-Aldrich) were used. Cyclocreatine (Ccr; also known as 2-imino-1-imidazolidineacetic acid), pCr, and phosphopyruvic acid (pPy) were purchased from Sigma-Aldrich. Recombinant CKB and pyruvate kinase (PK) were obtained from Acris (Herfold, Germany) and Calbiochem (San Diego, CA), respectively.

Proteome analysis. RC-rich membrane fractions of cells were isolated as described previously (2, 41). Briefly, cells were lysed in hypotonic buffer. After removing the nuclei, supernatants were treated with 1% NP-40 for 60 min, mixed with 70% sucrose, overlaid with 55 and 10% sucrose, and centrifuged at 38,000 rpm for 14 h. Proteins from membrane fractions were purified by using a 2D Clean-Up kit (GE Healthcare, Tokyo, Japan), followed by labeling with fluorescent dyes: Cy5 for replicon cells, Cy3 for parental cells, and Cy2 for the protein standard containing equal amounts of both cell samples. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was performed using Immobiline DryStrip as the first-dimension gel and 12.5% polyacrylamide gel as the second-dimension gel. The 2D-DIGE images were analyzed quantitatively using the DeCyder software (GE Healthcare). Student *t* test was performed on differences between the tested samples using DeCyder biological variation analysis module. Samples were analyzed in triplicate. The protein spots of interest were excised from the gel, subjected to in-gel digestion using trypsin or lysyl endopeptidase and analyzed by liquid chromatography (MAGIC 2002 System; Michrom Bioresources, Auburn, CA) directly connected to electrospray ionization-ion trap mass spectrometry (LCQ-decaXP; Thermo Electron Corp., Iwakura, Japan). The results were subjected to database (NCBInr) search by Mascot server software (Matrix Science, Boston, MA) for peptide assignment.

Plasmids. A human CKB cDNA (43; kindly provided by Oriental Yeast Corp., Tokyo, Japan) was inserted into the EcoRI site of pCAGGS, yielding pCAGCKB. To generate expression plasmids for HA-tagged versions of wild-type and deletion mutated CKB, the corresponding DNA fragments were amplified by PCR, followed by introduction into the BglII site of pCAGGS. A fragment representing the inactive mutant CKB-C283S was synthesized by PCR mutagenesis. To generate FLAG-tagged NS protein expression plasmids, DNA fragments encoding either NS3, NS4A, NS4B, NS5A, or NS5B protein were amplified from HCV strains NIHJ1 (1) and JFH-1 (23) by PCR, followed by cloning into the EcoRI-EcoRV sites of pcDNA3-MEF (20). To generate an HA-tagged NS3 expression plasmid, a fragment encoding NS3 with the HA tag sequence at its N terminus was inserted into pCAGGS.

siRNA transfection. The small interfering RNAs (siRNAs) targeted to CKB (CKB-1 [5′-UAAGACCUUCCUGGUGUGGTT-3′] and CKB-2 [5′-CGUCAC CCUUGGUAGAGUUTT-3′]) and the scramble negative control siRNA to CKB-2 (5′-GGCGUACUAGCUUAUUCGCTT-3′) were purchased from Sigma. Cells in a 24-well plate were transfected with siRNA using HiPerFect transfection reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The siRNA sequences for the other genes used in the siRNA screening are available upon request.

HCV infection. Culture media from Huh-7 cells transfected with in vitro-transcribed RNA corresponding to the full-length JFH-1 (47) was collected, concentrated, and used for the infection assay (3).

Quantification of HCV core protein and RNA. To estimate the levels of HCV core protein, aliquots of culture supernatants or of cell lysates were assayed by using HCV Core enzyme-linked immunosorbent assay kits (5). Total RNA was isolated from harvested cells using TRIzol (Invitrogen, Carlsbad, CA). Copy numbers of the viral RNA were determined by reverse transcription-PCR (RT-PCR) (2, 36, 46).

Immunoprecipitation, immunoblot analysis, and immunofluorescence microscopy. The analyses, as well as DNA transfection, were performed essentially as previously described (42). Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, 1 mM calcium acetate). For immunoprecipitation, supernatants of cell lysates were precipitated with anti-FLAG antibody and protein A-Sepharose Fast Flow beads (GE healthcare). For immunofluorescence microscopy, anti-CKB goat PAb and anti-NS4A MAb as primary antibodies and Alexa Fluor 555-conjugated donkey anti-goat immunoglobulin G (Invitrogen) and Alexa Fluor 488-conjugated rabbit anti-mouse immunoglobulin G (Invitrogen) as secondary antibodies were used and observed under an LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Immunoelectron microscopy. Postembedding immunostaining using the colloidal gold-labeling method was performed as described previously (38). Cells were fixed in 4% paraformaldehyde–1% glutaraldehyde at 4°C for 1 h. After dehydration through a graded series of ethanol, cells were embedded in LR White (London Resin Company, London, United Kingdom) and sectioned. After blocking, section grids were incubated with a mixture of anti-NS4A and anti-CKB antibodies at 4°C overnight, followed by treatment with a mixture of 18-nm colloidal gold-conjugated donkey anti-mouse immunoglobulin G and 12-nm colloidal gold-conjugated donkey anti-goat immunoglobulin G antibodies (Jackson Immunoresearch, West Grove, PA) at 4°C overnight. The sections were stained with uranyl acetate and observed under a transmission electron microscope.

Measurement of CK activity and cellular ATP level. Cells were lysed with passive lysis buffer (Promega, Madison, WI), and CK activities were measured based on Oliver methods (40), in which the activity of converting creatine phosphate and ADP to creatine and ATP was measured. ATP levels in cell lysates were measured by using a CellTiter-Glo luminescent cell viability assay (Promega).

RNA replication assays in permeabilized replicon cells and in vitro. The RNA synthesis assay using permeabilized replicon cells was based on a previously described method (33). Briefly, SGR-JFH1 cells were treated with 5 μg of actinomycin D/ml for 2 h, followed by permeabilization with 50 μg of digitonin/ml for 5 min. The resulting mix was incubated with 500 μM concentrations of ATP, GTP, and CTP; 10 μCi of UTP ([α-^{32}P]UTP); 50 μg of actinomycin D/ml; and 5 mM pCr with or without 20 U of CKB/ml for 4 h at 27°C. RNA was extracted by using TRIzol and analyzed by 1% formaldehyde agarose gel electrophoresis. The cell-free RNA replication assay was performed as described previously (2).

In vitro helicase assays. Helicase activity on double-stranded RNA (dsRNA) was investigated as described previously (11) with some modifications. The 5′ end of the release strand was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Ambion). The dsRNA substrate was obtained by annealing the labeled RNA with a template strand RNA at a molar ratio of 1:1. The helicase assay mixture contained 5 nM dsRNA, helicase enzyme (80 nM NS3 or NS3-4A [kindly provided by R. De Francesco]), 6 mM ATP, in the presence or absence of 20 U of CKB/ml in an assay buffer (25 mM MOPS-NaOH [pH 7.0], 2.5 mM dithiothreitol, 100 μg of bovine serum albumin/ml, 3 mM $MgCl_2$, 5 mM pCr, 2.5 U of RNase inhibitor/ml). After the helicase reaction, samples were electrophoresed in a native 8% polyacrylamide gel and autoradiographed.

To determine the effect of PK/pPy system on the helicase activity, PK and pPy were used instead of CKB and pCr. Helicase activity on dsDNA was measured based on homogeneous time-resolved fluorescence quenching using a Trupoint helicase assay kit (Perkin-Elmer, Waltham, MA) according to the manufacturer's instructions.

In vitro protease assay. In vitro HCV protease activity of NS3-4A or NS3 was analyzed by using a SensoLyteHCV protease assay kit (AnaSpec, San Jose, CA) according to the manufacturer's instructions.

RESULTS

Identification of host factors involved in HCV RNA replication by comparative proteomic analysis of DRM fractions and RNAi silencing. To identify host proteins involved in the HCV RC, proteome profiles of the RC-rich membrane fraction in Huh-7 cells harboring subgenomic replicon RNA derived from genotype 1b, N isolate (SGR-N) were compared to those of parental cells by 2D-DIGE. We confirmed that the DRM fraction obtained from SGR-N cells is functionally active in a

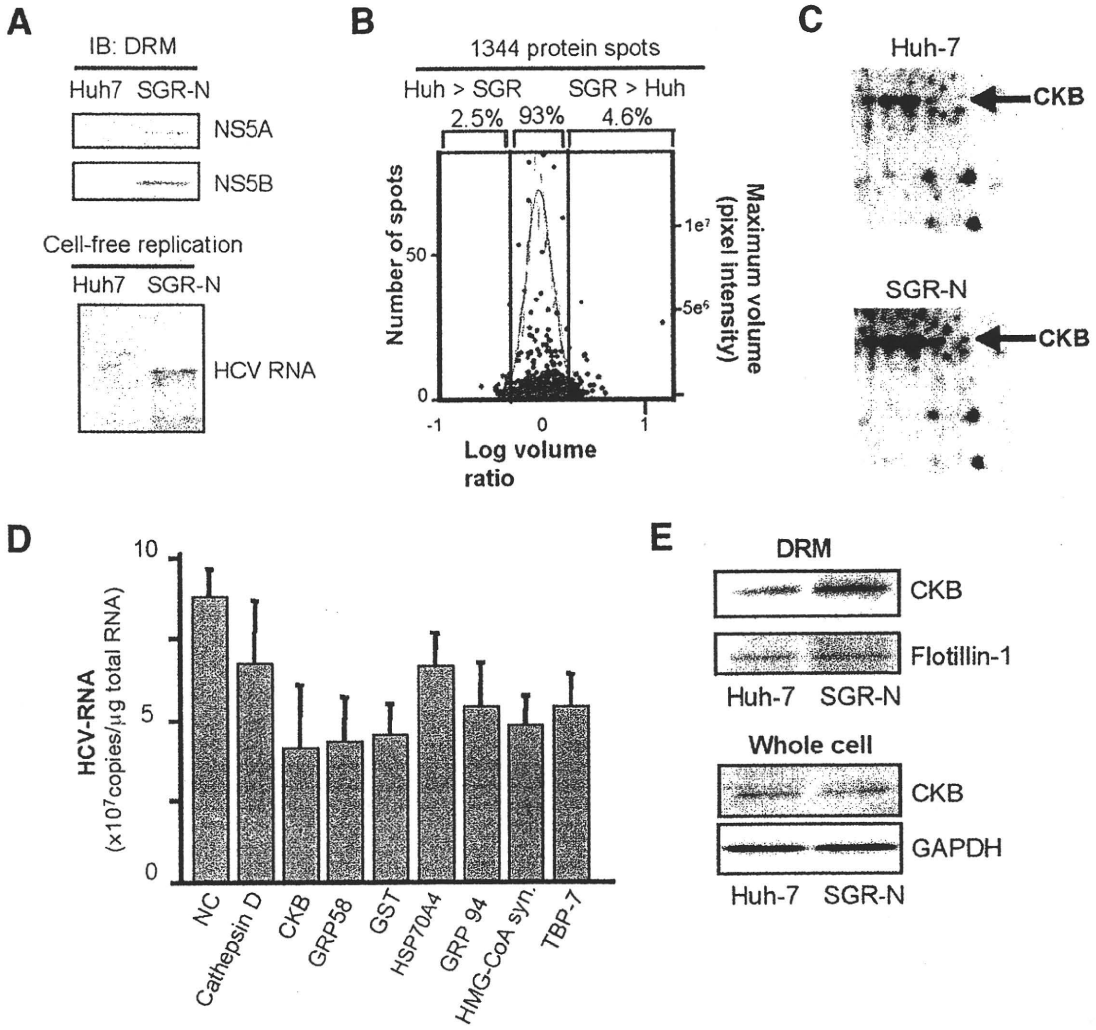

FIG. 1. Comparative proteomic analysis of DRM fractions and RNAi silencing. (A) Preparation of functionally active RC fraction for proteome analysis. DRM fractions obtained from SGR-N cells and parental Huh-7 cells were analyzed by immunoblotting with anti-NS5A and anti-NS5B antibodies (upper panel) and by the cell-free RNA replication assay (lower panel). (B) Histogram representation of proteins detected in 2D-DIGE. Images were analyzed quantitatively by the DeCyder software. The left and right *y* axis, respectively, indicate the spot frequency and the maximum volume of each spot, given against the log volume ratio (*x* axis). (C) Comparison of 2D-DIGE maps of proteins from DRM fractions of SGR-N cells and Huh-7 cells. Enlarged 2D-DIGE gel images of regions containing protein spots of CKB (arrows) are shown. (D) Effects of siRNAs of genes selected from comparative proteome analysis on HCV RNA replication. SGR-N cells were transfected with siRNA specific to cathepsin D, CKB (siCKB-1), GRP58, GST, Hsp70 protein 4, GRP94, HMG-coenzyme A synthase, or Tat binding protein 7 or with nontargeting (NC) siRNA. At 48 h posttransfection, total RNA was isolated and HCV RNA levels were assessed by real-time RT-PCR. (E) Enrichment of CKB in the DRM of HCV replicon cells. Equal amounts of DRM fractions from SGR-N and parental Huh-7 cells, or whole-cell lysates from both cells were analyzed by immunoblotting with antibodies against CKB, flotilin-1 or GAPDH.

cell-free replication assay (Fig. 1A). Three independent proteome experiments were performed for a reliable analysis of protein expression. Approximately 1,300 spots were resolved in each gel, and 4 to 5% of the protein spots represented a >2-fold increase in the membrane fraction of replicon cells in each experiment (Fig. 1B). The protein spots that exhibited high reproducibility (an example shown in Fig. 1C) were excised, digested by trypsin or lysyl endopeptidase, and analyzed by mass spectrometry, which identified the corresponding proteins in 27 cases (Table 1). Among the proteins implicated in a variety of functional categories, 10 were involved in protein folding, mainly as chaperones, 7 were metabolic and biosynthesis enzymes including proteins for redox regulation or energy pathways, 3 were involved in cytoskeleton organization, and 3 proteins were related to cellular processes, mainly proteolysis pathways. The viral NS proteins identified as differentially expressed proteins in the analysis were not listed.

In order to identify host factors involved in HCV replication, we examined the effects on viral RNA replication of transfection of SGR-N cells with siRNAs against genes encoding nine proteins belonging to diverse classes of biological functions (Table 1). Each siRNA reduced the HCV RNA level to 47 to 76% of the level of the siRNA control (Fig. 1D). None of the siRNAs tested exhibited considerable cytotoxicity against the replicon cells, ruling out overt toxicity as a mechanism for inhibition of viral RNA replication. Among the candidate