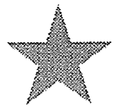


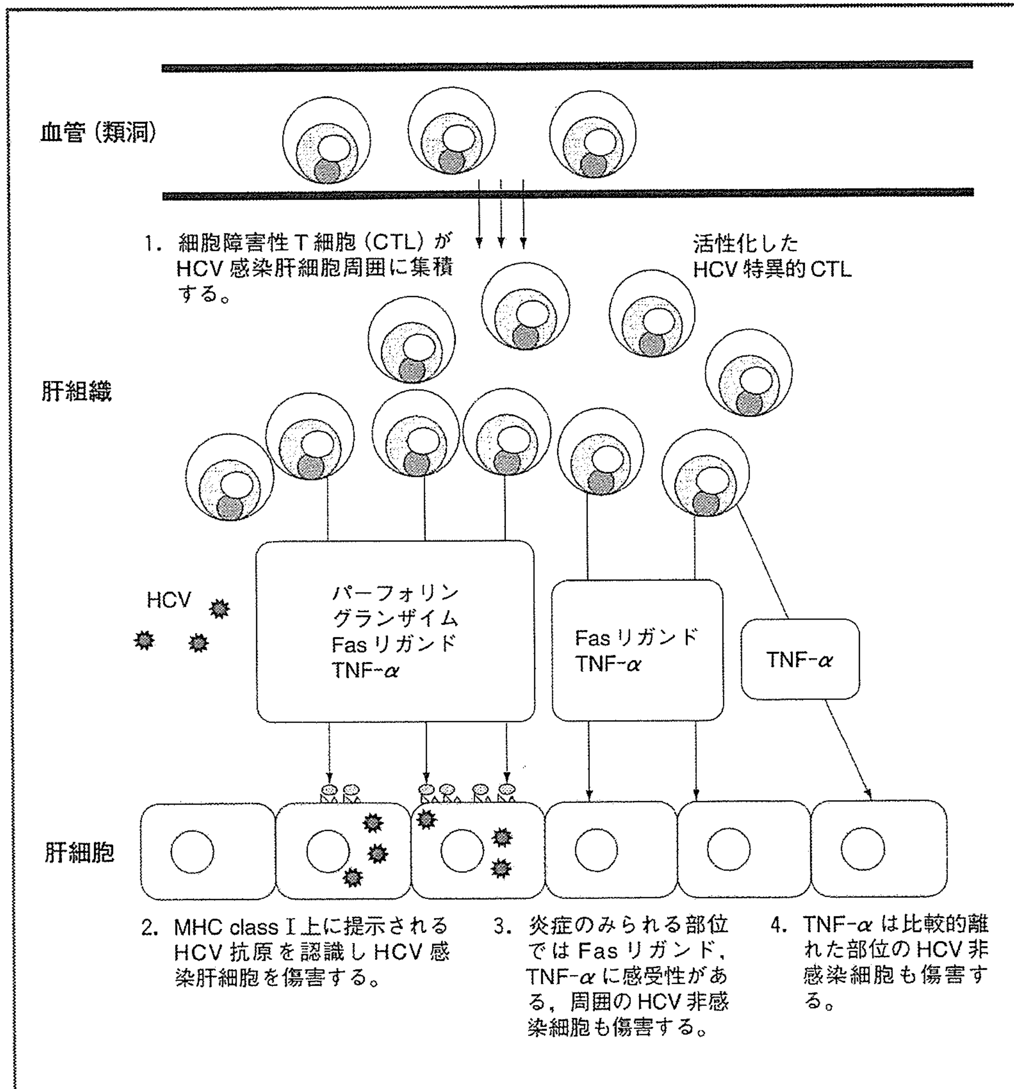


図3　HCV特異的細胞障害性T細胞による肝細胞傷害

持続感染に寄与しているとされている。しかし一方，チンパンジーでのHCV感染実験では，HVR1に変異はみられずに持続感染に発展しているとの報告もあり，E2領域の変異が感染の持続化に大きな役割を果たしているかを疑問視する意見もある。

2) 細胞性免疫からの逃避

HLA class Iあるいはclass II拘束性T細胞のエピトープの変異は，HCV感染肝細胞の排除を妨げることにより感染の持続化に寄与する可能性がある。これまでの研究では，感染HCVのクローニングにより，CD 8^{+} CTLの認識を妨げるアミノ酸変異が認められている。また，HLA-DRB1拘束性でNS3由来のペプチドを認識するTh1タイプのT細胞に対し，このエピトープ内の1つのアミノ酸に変異が起こると，分泌するサイトカインがTh1タイプからTh2タイプに

変化したことが報告されたが[15]，このようにTh 2タイプの免疫応答が生体内で優位になると持続感染が成立しやすくなると考えられる。

C型急性肝炎6例での検討では，HCVのCTLエピトープにおけるアミノ酸変異は3例にみられ，変異した配列はもとの配列と比較し，CTLに認識されにくく，さらにCTLを効果的に誘導できないと報告された[16]。CTLエピトープの変異は，より強いエフェクター機能を有するCTLをターゲットにして起こり，持続感染に寄与することも想定される。

一方，HCV感染に対する初期のCTL応答は多様であり，1つのエピトープの変異のみで持続化は説明できないとの指摘もある。エスケープミュータントは持続感染の原因というより，持続感染の結果をみている可能性も否定できない。

2．HCV感染による免疫細胞の機能抑制

抗HCV抗体は感染後2～4ヵ月間もの間出現せず，さらに抗体が現れてもHCV感染は持続し肝炎は進行する。また，細胞性免疫においても，急性期には多様なT細胞応答が認められるが，慢性化するとその応答は劇的に減弱してしまう。肝内には多数のHCV特異的CD 8^{+} T細胞が存在してはいるものの，HCVを排除できない。さらに，C型慢性肝炎患者にはB型肝炎ウイルスや細菌感染などの合併も多く，以前から生体での免疫力の低下が想定されてきた。近年，HCV自体が能動的に生体の免疫機構を抑制している可能性が考えられており，それを示唆する報告が多数なされるようになった。

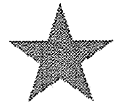
1）自然免疫に対する抑制

HCVのE 2蛋白は細胞表面上のCD 81と高い親和性をもち，CD 81はHCVが細胞感染する際の受容体となりうると考えられている。HCVのE 2蛋白はNK細胞上に発現するCD 81と結合し，直接NK細胞の機能を低下させる作用があることが報告された[17]。NK細胞はIFN治療1週間後には肝内への浸潤が観察されるが，治療有効群と治療無効群との間でNK細胞の細胞障害活性は異なり，IFN治療の有効性を予知する指標となるとされている。このことからも，NK細胞を中心とした自然免疫系もHCV排除には重要な役割を果たすと考えられるが，HCVが直接NK細胞の活性を抑制することは感染の持続化に対し大きな意味をもつ。

NK細胞の細胞障害活性低下の具体的な機序については，いくつかの報告がなされている。HCVコア蛋白は，*p53*依存性にtransporter associated with antigen processing 1（TAP 1）の発現を増強することで，MHC class I発現を増強する。MHC class Iの発現増強は，NK細胞のHCV感染肝細胞に対する細胞障害活性を低下させ，感染の持続化に寄与する可能性がある。また，C型慢性肝炎患者のNK細胞では，その活性を抑制するシグナルを伝えるレセプターであるCD 94/NKG 2 Aの発現が増強していると報告された[18]。さらに，HLA A 2拘束性T細胞のエピトープとして知られているHCVコア蛋白35-44のアミノ酸配列に相当するペプチドは，CD 94/NKG 2 AのリガンドであるHLA-Eと結合することによりHLA-Eの発現を安定化させて，NK細胞による障害を抑制し，HCVの持続感染に寄与する可能性も考えられている[19]。NK細胞の他の抑制性レセプターであるKIR 2 DL 3がHCVの排除に影響を及ぼすことも想定されている[20]。

2）液性免疫に対する抑制

C型肝炎患者の末梢リンパ球は，前述のCD 81分子が強発現しており，HCVが感染しやすい状態になっていることが考えられ，感染を介して抗体産生などに影響を及ぼしている可能性がある。また，他の感染実験でも，中和抗体の抗体価は低く再感染を防止することはできなかったため，B細胞応答もHCVにより抑制されていることが想



定されている。

3) T細胞に対する抑制

HCV特異的CTLのエフェクターとしての機能は明らかに低下している。C型慢性肝炎患者の末梢リンパ球においては，その機能の発現に重要なCD 3ζ鎖の発現が，また肝浸潤リンパ球においてはT細胞レセプターδ鎖やCD 56の発現が低下していることが報告されている。また，HBV特異的CTLに比しHCV特異的CTLは明らかにパーフォリンの発現量が少なく，これは機能低下を示す1つの例とされているが，このような免疫細胞自体の機能低下もC型肝炎の持続化に関与することが想定されている。C型急性肝炎時にはCCR 7$^-$ CD 8$^+$ T細胞(メモリー・エフェクター細胞)は細胞障害活性が低下しているが，これにIL-2を加えると完全にエフェクター機能を有する細胞になることから，T細胞が活性化する際のIL-2の欠乏がCTLの機能低下の主な原因であるとの報告もある[21]。循環しているコア蛋白がIL-2産生のシグナル伝達の抑制に関与していることも想定されている。

C型慢性肝炎患者において，HCV特異的CD 4$^+$ T細胞の存在は認められるものの抗原特異的な増殖能は抑制され，さらに後述するように，抗原刺激に特異的なIL-10やTGF-β産生も有意に認められており，Th細胞やCTLの十分な活性化が起こらずにHCVの持続感染を導くと考えられる。

また，HCV感染肝細胞より遊離し末梢血中に存在するHCVコア蛋白は，T細胞のgC 1 qRと結合することで，T細胞の増殖や活性，IFN-γ産生能を阻害することが報告された。HCVコア蛋白は血中にナノグラムの単位で存在しており，gC 1 qRと結合するには十分量と考えられるが，肝組織内ではさらに高濃度のコア蛋白が存在していると想定され，肝浸潤リンパ球に少なからず影響を与えていると推測される。

HCV NS 4 A/B蛋白は，細胞内で小胞体からゴルジ体への輸送を妨げることにより，HLA class I分子の細胞上への発現を抑制することが報告された[22]。これにより，HCV特異的CD 8$^+$ T細胞がHCV感染肝細胞を認識しにくくなり，HCVの感染持続化に繋がることも考えられる。

さらに，肝臓には類洞内皮細胞やKupffer細胞といった免疫に関与する細胞が存在するが，それらは成熟した樹状細胞とは異なり，ウイルス抗原は提示するもののCD 80やCD 86といった共刺激分子に乏しいため，T細胞を十分に刺激できないばかりか，かえって免疫寛容を誘導してしまうことも考えられている[23]。

4) 樹状細胞に対する抑制

樹状細胞は免疫を誘導するうえで，重要な役割を担っている。C型慢性肝炎患者においては，樹状細胞のallogeneicのT細胞を刺激する能力が低下していることや，HCVコアとE 1蛋白は樹状細胞の成熟化を抑制することでT細胞刺激能を減弱させていたことがこれまでに指摘されている。近年，C型慢性肝炎患者では形質細胞様樹状細胞の数やIFN-α産生能はいずれも低下し，一方，骨髄系樹状細胞ではfrequencyに差異は認められないものの，アロリンパ球の刺激能は低下していることが報告された[24]。この報告では，骨髄系樹状細胞の機能低下はHCVコア蛋白とNS 3蛋白で誘導可能であり，これらの蛋白の認識はTLR 2を介して行われるとされた。これは，ウイルス蛋白が樹状細胞の機能を低下させ，細胞免疫応答の誘導を抑制する1つのメカニズムになると考えられる。また，細菌やウイルス由来のTLRリガンド(病原体成分)により活性化した樹状細胞は，クロスプレゼンテーションの作用が弱まり，免疫応答が減弱することが報告されたが[27]，HCVにも同様の機構が存在していることも考えられる。実際，C型慢性肝炎患者の未成熟の樹状細胞では，健常者と比較してTLR 2の発現が低

下しており，TLR 2で刺激した樹状細胞はT細胞増殖効果が減弱していることも示された[28]。

樹状細胞によるNK細胞活性化の抑制も指摘されている。IFN-α刺激後に，樹状細胞はその表面にMHC class I-related chain A and B (MICA/B)を発現させ，NK細胞を活性化するが，C型慢性肝炎患者においてNK細胞が有効に活性化できないのは，type I IFNによるIL-15産生能が低下しており，MICA/B発現の増強が抑制されていることが原因であるとされた[25]。

HCVは，E 2蛋白が樹状細胞上に発現するDC-SIGNに結合することより樹状細胞にも感染することや，soluble E 2蛋白も樹状細胞と結合が可能であることも報告され，それらにより樹状細胞は機能低下に陥る可能性も考えられている。HCVコア，NS 3，NS 5 A，NS 5 B蛋白は成熟した樹状細胞にアポトーシスを誘導することも報告されている[26]。

一方，チンパンジーへの感染実験では，樹状細胞の機能低下は認められないとする結果や，C型慢性肝炎患者の検討で樹状細胞の成熟化やアロ刺激能は正常であることも報告されている[29]。HCVコアやNS 3蛋白を発現させたヒト樹状細胞は，炎症性サイトカインの産生やフェノタイプ，アロT細胞刺激能は正常であるとされた[30]。健常者と比較してC型肝炎患者では形質細胞様，骨髄系とも樹状細胞のfrequencyは低いが，循環血中の樹状細胞のフェノタイプや機能は低下しておらず，形質細胞様樹状細胞においてIFN-α産生能も1つひとつの細胞レベルでは低下していないとの報告もある[31]。また他の報告によると，HCVの急性肝炎では，末梢血中に形質細胞様樹状細胞が著明に減少しているが，それらは未成熟でHLA DRやCCR 7の発現やIFN-αの産生能が低下しているとする一方，慢性肝炎患者の樹状細胞では，形質細胞様樹状細胞に明確な変化は認められていない[32]。

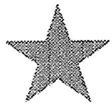
樹状細胞のC型肝炎への関与は今後さらなる検討が必要である。

5) HCV蛋白による細胞に対するその他の影響

HCVトランスジェニックマウスの系で，HCV蛋白がIFNによりもたらされる細胞内伝達シグナル(Jak-STAT系)を抑制することが示唆され，これがIFN不応性の一因となることが想定されている[33]。また，HCV蛋白は感染した肝細胞のFasを介したアポトーシスを抑制しており，これも持続感染を誘導するのに重要であると指摘している。一方，HCVコア蛋白はJurkat細胞に対しFasを介したアポトーシスの経路を活性化させるとの報告もある[34]。細胞内コア蛋白は，TNFレセプターの細胞内ドメイン(TNFRI)あるいはFasと結合し，細胞にアポトーシスを誘導することが証明され，肝細胞やリンパ球のアポトーシスに関与する可能性も考えられている。

3．HCV特異的制御性T細胞の関与

生体の免疫応答を抑制する要因の1つとして抗原を特異的に認識してIL-10やTGF-βを産生する制御性T (regulatory T：Tr)細胞が注目されている。C型慢性肝炎患者においては，Tr細胞と考えられるCD 4^{+} CD 25^{+} T細胞のfrequencyが高く，この細胞集団は直接T細胞の機能を抑制し，これがHCV特異的細胞性免疫の質的，量的な抑制を引き起こして，肝炎の持続化に寄与していると想定されている[35](図4)。HCVコア蛋白に特異的なTr細胞がC型慢性肝炎の末梢血から分離誘導され，この細胞が産生するIL-10がHCV感染の持続化に関与すると報告された[36]。また，C型肝炎患者の肝内にはIL-10を産生するHCV特異的CCR 7^{-} CD 8^{+} Tr細胞が存在し，肝内に多数集積しているHCV特異的CCR 7^{-} CD 8^{+}メモリーT細胞の機能を抑制することも報告された[37]。さらに，HCV NS 4蛋白は，C型肝炎患者のみならず，健常者の末梢単核球からもIL-10の産生を促しIL-12の分泌を抑制させ，さらに樹



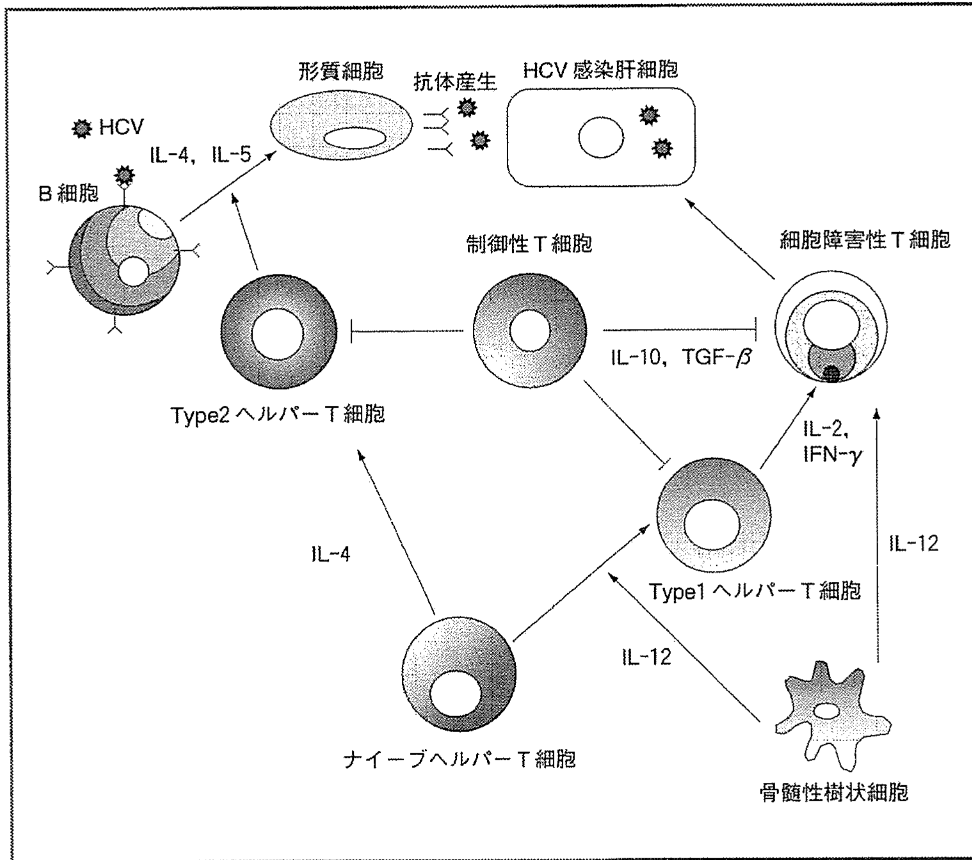


図4 HCV に対する免疫細胞の誘導と制御性 T 細胞による免疫抑制

状細胞の分化成熟化を抑制するとの報告もあり，細胞性免疫の活性化抑制の１つの機序として興味深い[38]。

以上のように Tr 細胞の HCV 感染持続化への関与が強く示唆されているが，まだ不明な点も多くさらなる検討が必要である。

４．その他の HCV 持続感染の機序

非構造蛋白領域である NS５A の C 末端側に存在する 40 個のアミノ酸からなる部分(IFN-sensitivity determining region)の変異は，IFN により誘導され，抗ウイルス効果を発揮するプロテインキナーゼと結合し，その活性化を阻害することにより IFN 治療抵抗性を得るとされている。また，HCV NS 3/4 のセリンプロテアーゼは，細胞が抗ウイルス効果を発揮するうえで重要な interferon regulatory factor-3 を抑制するといわれている。近年，HCV NS 3/4 のセリンプロテアーゼは，二本鎖 DNA の受容体である retinoic acid inducible gene I(RIG-I)と抗ウイルスシグナルとを結ぶアダプター蛋白質である Cardif を標的とし，これを不活化させることにより細胞の抗ウイルス効果を抑制していることが報告された[39]。また，分子レベルではウイルスが感染細胞に，ウイルス遺伝子発現抑制，抗原のプロセッシング抑制といった影響を与えていることも想定されている。

HCV 感染に対する免疫を介した治療

IFN-α は現在，HCV を生体から排除すること

が可能な唯一の治療薬である。低濃度のIFN-αは，HCVコア蛋白をターゲットにしたDNAワクチンにより誘導される細胞性免疫応答を3～4倍増強するが，高濃度では逆に，CTL応答を抑制すると報告された[40]。C型肝炎治癒症例はTh 1優位であるといわれているが，近年，C型慢性肝炎の治療に用いられているリバビリン(商品名レベトール®)は，患者の免疫応答をTh 2からTh 1優位に変化させることが，抗ウイルス効果の1つの機序と考えられている。

IFN治療によりSVRが得られた症例ではRelapse例や無効例に比し，肝内や末梢のHCV特異的CTL活性が強くみられることも報告されている[41]。このなかで筆者らは，IFNとリバビリンの併用療法は，CTL応答を増強してHCVを排除するというより，治療前から存在するCTL応答がIFN・リバビリン併用療法による直接の抗ウイルス作用を増強して排除するのではないかと述べている。

樹状細胞にNS 3蛋白をパルスし，CpGオリゴヌクレオチドで成熟させるとCD 40の発現が増強し，強い免疫応答を誘導することができ，マウスでHCV NS 3を発現させたワクシニアウイルスの感染を抑制できたことから[42]，樹状細胞を用いた細胞免疫療法の開発も今後期待される。

おわりに

肝炎動物モデルや臨床検体の解析などにより，ウイルス肝炎における免疫応答が長年にわたり研究されてきた。肝細胞障害にはCTLを中心とした細胞性免疫応答の関与が明らかになり，その障害機序や生体免疫応答の抑制機序も徐々に解明されてきている。免疫応答を適切にコントロールすることは，肝炎ウイルス排除あるいは肝炎の鎮静化に重要であるが，これから解明していかなければならない問題も多々ある。これらの問題点を生体免疫反応のみならず，ウイルス側からも詳細に解明していくことで，将来，肝炎ウイルスを完全に生体から排除できる治療法の開発が可能になると考えられる。今後のウイルス学，免疫学のさらなる発展を期待する。

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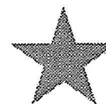

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Hepatitis C Virus Core Functions as a Suppressor of Cyclin-dependent Kinase-activating Kinase and Impairs Cell Cycle Progression*

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We investigated how the hepatitis C virus (HCV) core protein affects the cell cycle profile and cell cycle-related molecules by using the HCV core-expressing stable transfectant. Analysis of the cell cycle profile showed that HCV core impaired G_1 to S transition. The E2F-mediated transcription, phosphorylation of the retinoblastoma protein, and cyclin-dependent kinase (CDK) 4 and CDK2 activities were suppressed in HCV core-expressing cells. The expression levels of G_1 phase-related CDKs/cyclins and various CDK inhibitors were not substantially affected by expression of HCV core. When influences of HCV core on CDK-activating kinase (CAK) were examined, the expression levels of the CAK components, CDK7, cyclin H, and MAT1, were not affected. However, formation of the ternary CAK complex, CAK activity, and the CDK2 level with activating phosphorylation were inhibited by expression of the HCV core. The direct effect of HCV core on CAK was further assessed in the cell-free system by adding the *in vitro* translated HCV core protein to the anti-CDK7 immunoprecipitate from the cell. The results showed that HCV core led to dissociation of MAT1 from the CAK complex and suppressed the CAK activity. Furthermore, the binding assay revealed that the HCV core was directed against CDK7. Their interaction occurred mainly in the nucleus by the immunostaining. In conclusion, the HCV core protein interacts with CAK and functions as an extrinsic suppressor of CAK. This may be the molecular basis of HCV core-mediated suppression of cell cycle progression. Our findings suggest a novel mechanism concerning HCV core-mediated alteration in the cell cycle machinery.

Hepatitis C virus (HCV)[1] is a major etiologic cause of acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1). HCV is an enveloped virus with a plus-stranded RNA genome of about 9.5 kb in length (2). At least 10 HCV proteins are generated from proteolytic processing of a single large polyprotein precursor in the following order, NH_2-core-envelope 1-envelope 2-p7-nonstructural 2-nonstructural 3-nonstructural 4A-nonstructural 4B-nonstructural 5A-nonstructural 5B-COOH (3, 4). A series of studies have revealed that, among HCV proteins, the HCV core protein substantially affects cellular functions, which may be relevant to the pathogenesis of HCV-related liver diseases. Persistent expression of the HCV core has been reported to lead to malignant transformation of the host cell *in vitro* (5, 6) and *in vivo* (7). HCV core has also been shown to modify the cellular apoptotic cascade under various stimuli (8–11). In addition, the HCV core has been demonstrated to modulate various cellular signal transduction pathways (6, 10, 12–15). Such biological activities of the HCV core to the host cell are thought to be triggered by its direct interaction with cellular proteins. More than 10 HCV core-binding proteins have so far been identified (6, 10, 14–23).

The eukaryotic cell cycle progression is tightly regulated by the cyclin-dependent kinases (CDKs) and cyclins (reviewed in Refs. 24–28). Various CDK-cyclin complexes work in different phases during cell cycle progression; CDK4/6-cyclin D (cycD) plays a major role in the mid-G_1 phase, CDK2-cycE plays a role in the late G_1 phase, CDK2-cycA plays a role in the S phase, and CDC2 (also termed as CDK1)-cycB plays a role in the G_2/M phase. In particular, the retinoblastoma protein pRB is important as a substrate of the CDK-cyclin complexes in the G_1 phase. Once, the complexes of CDK4/6-cycD and CDK2-cycE phosphorylate pRB, the transcription factor E2F is activated. Then the E2F enhances transcription of many genes to enter the S phase. The functions of CDK-cyclin complexes are negatively regulated by CDK inhibitors, which are divided into two groups, a CIP/KIP family ($p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$) and an INK4 family ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$). $p21^{CIP1}$ is an especially well known transcriptional target of the tumor suppressor p53 (29).

The CDK activity is also controlled by both inhibitory and activating phosphorylations (25, 26). The amino-terminal inhibitory phosphorylation sites (Thr-14 and Tyr-15 in human CDK2) are dephosphorylated by CDC25 phosphatases. On the other hand, phosphorylation of a conserved threonine residue in the T-loop (Thr-160 in human CDK2) is required for full activation of CDKs. The CDK-activating kinase (CAK) undergoes the activating phosphorylation of CDKs. CAK is composed of three components, a catalytic subunit CDK7, a regulatory subunit cycH, and an assembly factor MAT1 (30–32). It has been shown that CAK can phosphorylate all cell cycle-related CDKs, CDC2, CDK2, CDK3, CDK4, and CDK6, at least in the cell-free system (30–34).

Thus far, the effects of HCV core on the cell cycle profile and cell cycle-related molecules have been studied by a few investigators (21, 22, 35). It has been reported that the HCV core can

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[1] The abbreviations used are: HCV, hepatitis C virus; CDK, cyclin-dependent kinase; cycD, cyclin D; CAK, CDK-activating kinase; aa, amino acids; GST, glutathione *S*-transferase; CTD, carboxyl-terminal domain; CL2, BNL CL.2; IP, immunoprecipitation; MOPS, 4-morpholinepropanesulfonic acid.

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bind to both p53 and p21^{CIP1} and that its binding to p53 up-regulates the p53-dependent transcription activity of p21^{CIP1} in cultured cells transiently transfected with HCV core-expressing plasmid (21, 22). The stable transfectant with HCV core-expressing plasmid derived from Chinese hamster ovary cells has also been shown to impair the cell cycle regulation accompanied by enhancement of p21^{CIP1} expression (35). However, molecular mechanisms concerning HCV core-mediated modulation on the cell cycle-related molecules other than the p53/p21^{CIP1} system have not been elucidated. To solve this, we investigated the effects on cell cycle-related molecules caused by persistent expression of the HCV core in cultured murine normal liver cells. In this process, we found that CAK is a novel target of the HCV core and that the HCV core suppresses cell cycle progression by a direct inhibitory effect on CAK assembly and activity.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The HCV core-expressing plasmid pc/3EFΔNCTH, which was constructed from a mammalian expression vector pc/3EFpro, carried the whole HCV core gene and the 5′-part of the envelope 1 gene of a genotype 1b HCV strain (36). Both pc/3EFpro and pc/3EFΔNCTH were kindly provided by Dr. T. Wakita (Department of Microbiology, Tokyo Metropolitan Institute of Medical Science). pF/core(1–191) was synthesized from pCMVtag2B (Stratagene) by inserting the HCV core gene (amino acids (aa) 1–191) downstream of the T3 promoter and used for the *in vitro* translation. pTALluc (Clontech) encoded the luciferase gene driven by the minimal TAL promoter. pE2Fluc (Clontech) was a derivative of pTALluc and possessed a repeated sequence of the E2F-responsive element upstream of the TAL promoter. pCMVβ (Clontech) expressed the β-galactosidase gene driven by the cytomegalovirus promoter. Plasmids p5GEX/hCDK2, p5GEX/hCDK7, p5GEX/hcycH, and p5GEX/hpRB(379–793) were used for the production of human CDK2, CDK7, cycH, and pRB as glutathione *S*-transferase (GST) fusion proteins. To construct these plasmids, whole coding regions of the cDNAs for CDK2, CDK7, and cycH and the part of the pRB cDNA (corresponding to aa 379–793 of pRB) were obtained by PCR using a cDNA sample from human hepatoma-derived cell lines, Huh-7 (37) (for CDK2) and HepG2 (38) (for pRB), or using the human adult liver cDNA library (Clontech) (for CDK7 and cycH). The cDNA fragments were cloned into the multicloning site of the plasmid p5GEX-1 (Amersham Biosciences). Plasmid p5GEX/core(1–122) expressed the fusion protein of GST with the truncated HCV core protein (aa 1–122). Plasmid pGCTD produced the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II fused to GST (39), which was a kind gift from Dr. William S. Dynan (Institute of Molecular Medicine and Genetics, Medical College of Georgia).

Cell Culture and Protein Extraction—A murine normal liver cell line, BNL CL.2 (CL2) (40), and a human hepatoma cell line, HepG2 (38), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml of streptomycin sulfate, 100 units/ml of penicillin G sodium, and 0.25 μg/ml of amphotericin B at 37 °C in a 5% CO_2. Three independent clones of the HCV core-expressing cells (designated as CL2 core-I, -II, and -III) and the negative control cells (mock) were established from the CL2 cells as described elsewhere (15, 41). The total cellular protein was extracted with the lysis buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.1% 2-mercaptoethanol, 1% Triton X-100, 10 mM β-glycerophosphate, 0.5 mM sodium vanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride and used for Western blotting, immunoprecipitation (IP), kinase assay, and *in vitro* binding assay. The nuclear fraction was obtained from the cells by the method described elsewhere (42).

Analyses of Cell Growth—To examine the cell growth curve, 5×10^3 of CL2 mock and core cells were seeded on a 96-well culture plate. After 24, 48, 72, and 96 h, the net number of viable cells were assessed colorimetrically using the water-soluble tetrazolium (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2*H*-tetrazolium monosodium salt) (Roche Applied Science) (43). This assay is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenase in viable cells. For the assay, 10 μl of the water-soluble tetrazolium reagent was added to the 100-μl culture medium, followed by the incubation at 37 °C for 1 h. Then the optical density at 450 nm was measured. The assay was done in quadruplicate, and the values were expressed as the means ± S.D.

Analysis of Cell Cycle Profile—The flow cytometric analysis to examine the cell cycle profile was done by the method described previously (44) with minor modifications. Briefly, the CL2 mock and core cells were fixed with 70% ethanol and stored at −20 °C. After centrifugation, the pellet was resuspended in the phosphate-buffered saline and incubated for 30 min in the presence of 0.2 mg/ml of propidium iodine and 1 mg/ml RNase A to stain the cellular double-stranded nucleic acid. After filtration with a 60-μm mesh filter, the cell suspension was analyzed on a FACScaliber flow cytometer and with Cellquest software (Becton Dickinson). Percentages of cells representing G_0/G_1, S, and G_2/M phases were determined.

Reporter Gene Assay—For cotransfection analysis, 8.0×10^4 of CL2 cells were seeded in a 35-mm-diameter culture dish and cotransfected of 0.5 μg of the reporter plasmid (pE2Fluc or pTALluc) with 0.5 μg of the effector plasmid (pc/3EFpro or p/3EFΔNCTH) using 6 μg of the cationic liposome (Lipofectin; Invitrogen). For the assay using cells constitutively expressing the HCV core, 8.0×10^4 of CL2 mock or 1.2×10^5 of CL2 core cells were seeded and transfected with 1 μg of the reporter plasmid (pE2Fluc and pTALluc). In all of the reporter gene assays, 0.1 μg of pCMVβ was also transfected. Two days after transfection, the cells were lysed and subjected to the luciferase and β-galactosidase assays. The luciferase activity was normalized for transfection efficiency based on the result of β-galactosidase assay. To determine the E2F-mediated transcription activity, the ratio of the fold activity in transfection with the pE2Fluc to that in transfection with pTALluc was calculated. All of the assays were done in quadruplicate, and the values were expressed as the means ± S.D.

Northern Blot Analysis—For Northern blot analysis, total cellular RNA was extracted from CL2 mock and core cells using a TRIZOL reagent (Invitrogen) based on the guanidine-isothiocyanate method. The poly(A)$^+$ RNA was selected from 50 μg of the total cellular RNA with an oligo(dT) column (Roche Applied Science). The sample was electrophoresed, transferred to a nylon membrane (Hybond N; Amersham Biosciences), and hybridized to the cDNA probe. After washing, the membrane was autoradiographed. The membrane was dehybridized by boiling in 0.5% SDS and further used for the hybridization to detect β-actin mRNA as a loading control (data not shown).

Antibodies—An antibody to pRB was obtained from Pharmingen. Antibodies to CDK4, CDK6, cycD1, CDK2, cycE, cycA, CDC2, cycB1, p53, p21^{CIP1}, p27^{KIP1}, CDK7, cycH, and MAT1 were purchased from Santa Cruz. An antibody to phosphorylated CDK2 at the Thr-160 residue (pT^{160}-CDK2) came from Cell Signaling. An antibody to GST was from Amersham Biosciences. A mouse monoclonal antibody to HCV core (45) was kindly provided by Dr. T. Wakita (Department of Microbiology, Tokyo Metropolitan Institute of Medical Science).

Immunofluorescence Staining and Confocal Microscopy—The CL2 core-I cells were plated on a two-well chamber slide. One day after seeding, the cells were fixed with 3% paraformaldehyde, 2% sucrose in phosphate-buffered saline for 30 min and permeabilized with ice-cold methanol for 3 min. After the blocking reaction with 10% fetal calf serum for 30 min at room temperature, the cells were incubated at 4 °C for 14 h with two primary antibodies, a mouse monoclonal HCV core antibody, and a rabbit polyclonal CDK7 antibody. Bound primary antibodies were revealed by incubation for 30 min at room temperature with Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) and Cy3-conjugated anti-rabbit IgG (Jackson Immunoresearch). Finally, a coverslip was mounted in the mounting medium (Vectashield; Vector Laboratories) with 4′,6-diamidino-2′-phenylindole-dihydrochroride, and the cells were examined by the confocal microscopic analysis using a Radiance 2100 BLD system (Bio-Rad). Colocalization of green and red signals in a single pixel produces yellow, whereas separated signals remain green or red.

Western Blot Analysis and Immunoprecipitation—For Western blot analysis, the total cellular protein was fractionated by SDS-polyacrylamide gel electrophoresis and blotted onto a membrane. After blocking with milk, the membrane was incubated with the specific antibody, followed by further incubation with a secondary antibody. Finally, the immune complex was detected by an enhanced chemiluminescent assay (Super Signal; Pierce). As for the IP reaction, 250–500 μg of the total cellular protein was precleared with protein A-Sepharose beads (Amersham Biosciences) at 4 °C for 1 h. After centrifugation, the supernatant was incubated with the specific antibody at 4 °C for 1–2 h. Then the beads was added, and the sample was further incubated at 4 °C for 1 h. After the extensive washing, the product was used for Western blot analysis and the kinase assay. In some experiments, the binding reaction of the IP product with the *in vitro* translation product was carried out at 4 °C for 3 h, prior to the subsequent experiments. The *in vitro*

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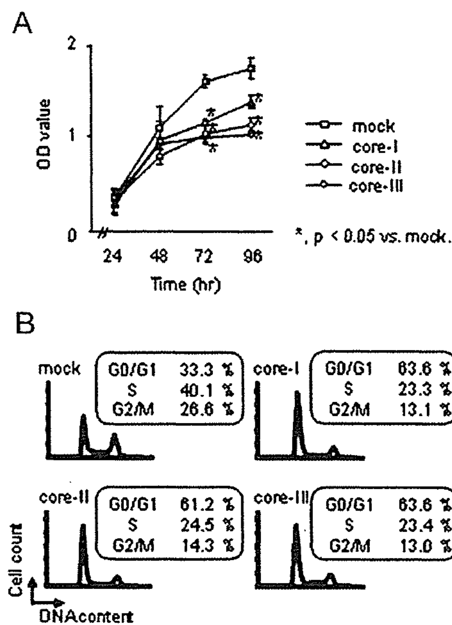

FIG. 1. **Cellular proliferation and cell cycle profile in CL2 mock and core cells.** *A*, CL2 mock and core cells were seeded on a 96-well culture plate, and viable cells were assessed by the water-soluble tetrazolium colorimetric assay. *B*, CL2 mock and core cells were incubated with propidium iodine and subjected to flow cytometry to examine the cell cycle profile.

translation was conducted using the TNT T3 coupled rabbit reticulocyte lysate system (Promega).

Synthesis for GST Fusion Proteins and Binding Assay—To construct various GST fusion proteins, the *Escherichia coli* BL21 (Stratagene) was transformed with p5GEX/hCDK2, p5GEX/hCDK7, p5GEX/hcycH, p5GEX/hpRB(379–793), p5GEX/core(1–122), pGCTD, and the empty plasmid p5GEX-1. The bacteria were grown in the medium containing 100 μg/ml of ampicillin sodium, and the proteins were expressed by the addition of isopropyl-1-thio-β-D-galactopyranoside. After sonication of the bacterial pellet, the proteins were purified with glutathione-Sepharose 4B beads (Amersham Biosciences). For the binding assay, ~5 μg of the various GST fusion protein or GST protein (negative control) was bound to the beads and incubated with 150 μg of the cellular lysate or 10 μl of *in vitro* translated protein at 4 °C for 2 h. After the extensive washing, the bound protein was used for Western blotting.

Kinase Assay—For the kinase assay, 250–500 μg of the total cellular protein was immunoprecipitated with CDK4, CDK2, and CDK7 antibodies as above. After extensive washing, the precipitate was subjected to the kinase assay in the presence of 12.5 mM MOPS, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 20 mM β-glycerophosphate, 1 mM NaF, 1 mM sodium vanadate, 5 mM dithiothreitol, 100 μM ATP, and 10 mCi of [γ-^{32}P]ATP in a total volume of 30 μl. Also added as a substrate was 2 μg of the GST-pRB fusion protein for the CDK4 kinase assay, the histone H1 (Calbiochem) for the CDK2 kinase assay, the GST-CDK2 fusion protein for the CAK assay, or the GST-CTD fusion protein for the CTD kinase assay. The reaction was carried out at 30 °C for 30 min. After the elution, the supernatant was fractionated by SDS-PAGE, and the gel was dried and autoradiographed. As for the CAK assay, the kinase reaction was carried out without [γ-^{32}P]ATP, and the phosphorylated product was detected by Western blotting using a pT^{160}-CDK2 antibody. All kinase assays were carried out with scaling up by 1.3–1.5-fold. Before the kinase reaction, the sample was divided into two tubes, and the remaining product was immunoblotted with the same antibody as that used for the IP reaction as a loading control (data not shown).

Statistical Analysis—Statistical analysis was performed using Student's nonpaired *t* test as appropriate. *p* values less than 0.05 were considered to be statistically significant.

RESULTS

HCV Core Protein Impairs G_1/S Transition in the Cell Cycle through Suppression of CDK4 and CDK2 Activities—Fig. 1*A* shows the cellular growth curve of CL2 mock and core cells.

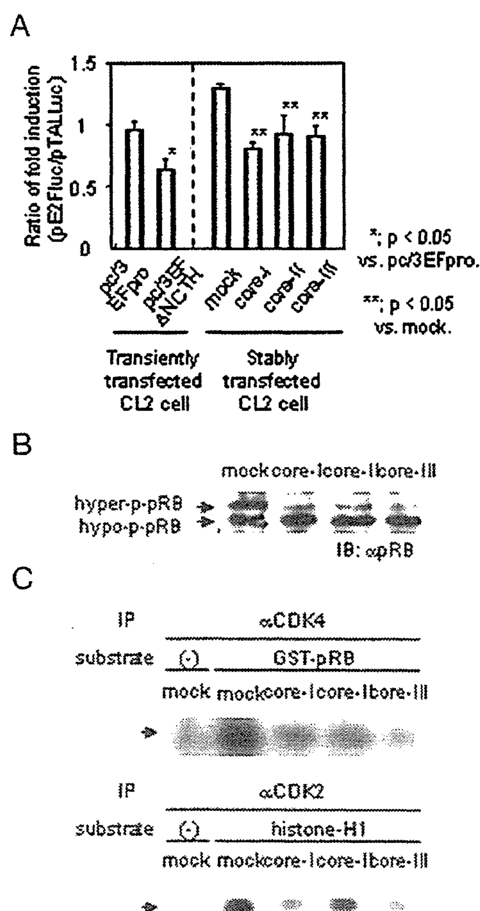

FIG. 2. **E2F-mediated transcription, pRB phosphorylation, and CDK4 and CDK2 kinase activities in CL2 mock and core cells.** *A*, CL2 cells were cotransfected of the reporter plasmid (pE2Fluc or pTALluc) with the effector plasmid (pc/3EFpro or p/3EFΔNCTH) (*left panel*). For the assay using CL2 mock and core cells, the cells were transfected with the reporter plasmid (pE2Fluc and pTALluc) (*right panel*). The E2F-mediated transcription activity was then assessed by the luciferase assay. The ratio of the fold activity in transfection with the pE2Fluc to that in transfection with pTALluc (pE2Fluc/pTALluc) was regarded as the E2F-mediated transcription activity. *B*, phosphorylation status of pRB was examined in CL2 mock and core cells by Western blotting. *C*, cellular proteins from CL2 mock and core cells were precipitated with a CDK4 or CDK2 antibody, and the precipitate was used for the kinase reaction with the GST-pRB fusion protein (for the CDK4 kinase assay, *upper panel*) or the histone H1 (for the CDK2 kinase assay, *lower panel*) as a substrate. *IB*, immunoblot.

Cell growth was significantly suppressed in the CL2 core cells, compared with the mock cells. Their cell cycle profiles were then assessed by flow cytometry (Fig. 1*B*). In the mock cells, ~30% of the cells were in the G_0/G_1 fraction, whereas in the CL2 core cells, more than 60% of cells were in this phase. The cells representing S and G_2/M phases were decreased by expression of the HCV core. The effect of the HCV core on the E2F-mediated transcription activity was next studied (Fig. 2*A*). In the cotransfection experiment using the CL2 cells, cotransfection of the HCV core-expressing plasmid pc/3EFΔNCTH significantly reduced the E2F-mediated transcription activity, compared with that of the negative control plasmid, pc/3EFpro. Significant reduction in E2F-mediated transcription by HCV core was also seen in the experiment using the CL2 mock and core cells. When the phosphorylation status of pRB was compared between CL2 mock and core cells (Fig. 2*B*), expression of HCV core caused a decrease of the hyperphosphorylated pRB

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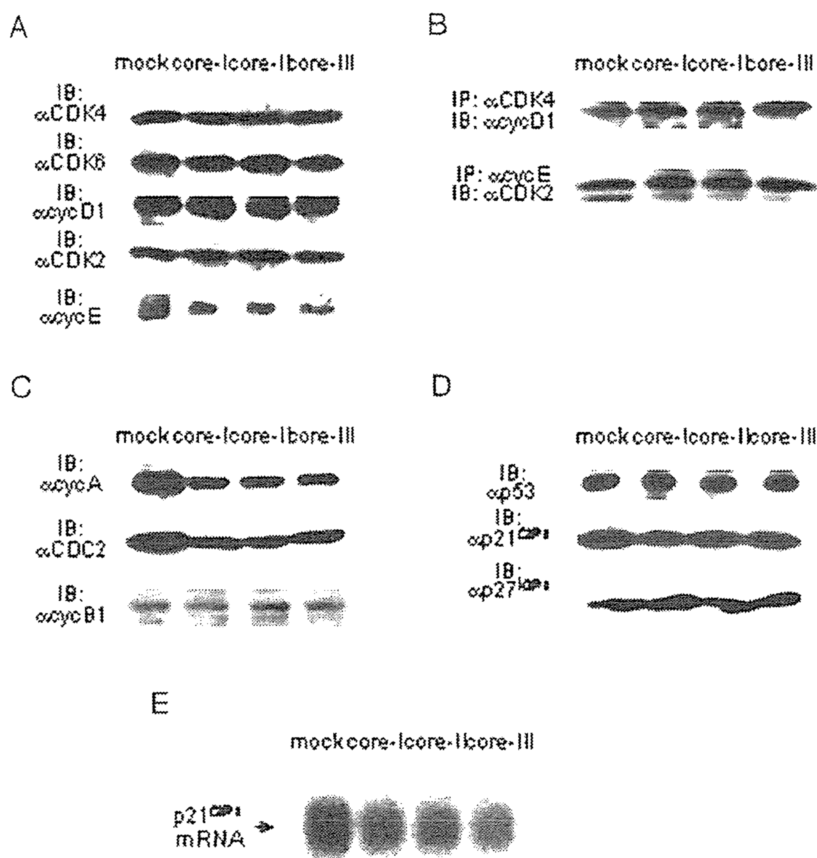

FIG. 3. **Expression levels of cell cycle-related CDKs/cyclins and CDK inhibitors in CL2 mock and core cells.** *A*, the expression levels of CDK4, CDK6, cycD1, CDK2, and cycE were examined in CL2 mock and core cells by Western blotting. *B*, the complexes of CDK4-cycD1 and CDK2-cycE were detected in CL2 mock and core cells by the IP/Western blot analysis. *C*, the expression levels of cycA, CDC2, and cycB1 were examined in CL2 mock and core cells by Western blotting. *D*, the expression levels of p53, $p21^{CIP1}$, and $p27^{KIP1}$ were examined in CL2 mock and core cells by Western blotting. *E*, the expression level of $p21^{CIP1}$ mRNA was examined in CL2 mock and core cells by Northern blotting. *IB*, immunoblot.

and an increase of the hypo-phosphorylated pRB. As for CDK4 and CDK2 kinase activities (Fig. 2*C*), both kinase activities were substantially lower in the CL2 core cells than in the mock cells. Thus, persistent expression of the HCV core impaired the G_1/S transition in the cell cycle, which may be due to the decreased CDK4 and CDK2 activities and the subsequently occurring inhibition of pRB phosphorylation and E2F-mediated transcription.

HCV Core Protein Does Not Influence the Expression Levels of G_1 Phase-related CDKs, Cyclins, and CDK Inhibitors—The expression levels of various CDKs and cyclins were studied in CL2 mock and core cells. When the G_1 phase-related CDKs and cyclins were examined (Fig. 3*A*), the levels of CDK4, CDK6, cycD1, and CDK2 did not differ between CL2 mock and core cells. Also, the expression level of cycE was not substantially affected by expression of the HCV core. The levels of both CDK4-cycD1 and CDK2-cycE complexes, as examined by the IP/Western blot analysis, were not decreased by HCV core (Fig. 3*B*). As for the expression levels of cycA, CDC2, and cycB1 (Fig. 3*C*), cycA and CDC2 were expressed more weakly in the CL2 core cells than in the mock cells. The cycB1 level did not differ between CL2 mock and core cells. When the expression levels of tumor suppressor p53 and its transcriptional target $p21^{CIP1}$ (29) were examined in CL2 mock and core cells, the p53 expression did not differ between them (Fig. 3*D*, *top panel*). The CL2 core cells showed slightly lower expression levels of $p21^{CIP1}$ mRNA than the mock cells (Fig. 3*E*), although its protein level was not much affected by HCV core (Fig. 3*D*, *middle panel*). Also, the $p27^{KIP1}$ expression was not different between CL2 mock and core cells (Fig. 3*D*, *bottom panel*). When the expression levels of proteins belonging to an INK4 family were determined by Western blot analyses, $p16^{INK4A}$, $p15^{INK4B}$, and $p18^{INK4C}$ were faintly detected in CL2 mock and core cells with no substantial differences between them. The $p19^{INK4D}$ expression did not differ between CL2 mock and core cells (data not shown). According to these results, HCV core suppressed the expression levels of cycA and CDC2, which play a role in the S and G_2/M phases, but did not influence the expression levels of G_1 phase-related CDKs, cyclins, and CDK inhibitors. The reduced expression of cycA and CDC2 may occur as a secondary effect of the decreased E2F-mediated transcription, because these two genes are known to be regulated by E2F (46, 47).

HCV Core Expression Results in Suppression of CAK Assembly and Activity—We further investigated the level of activating phosphorylation in CDK2 and the degrees of CAK expression, assembly, and activity in CL2 mock and core cells. The level of pT^{160}-CDK2 was significantly lower in the CL2 core cells than in the mock cells (Fig. 4*A*). For the expression levels of CAK components, CL2 core cells expressed the same levels of CDK7, cycH, and MAT1 as the mock cells. (Fig. 4*B*). For the complex formation of CAK examined by the IP/Western blot analysis, the level of the CDK7-cycH complex did not differ between CL2 mock and core cells (Fig. 4*C*, *top panel*). When the ternary form of CAK (CDK7-cycH-MAT1 complex) was investigated by means of the IP reaction using the mixture of CDK7 and cycH antibodies followed by the immunoblot using a MAT1 antibody, CL2 core cells displayed a lower level of ternary CAK complex formation than the mock cells (Fig. 4*C*, *bottom panel*). A similar result was also observed in the experiment with the IP reaction using a CDK7 or cycH antibody alone followed by the immunoblot with a MAT1 antibody (data not shown). The kinase assays using the GST-CDK2 substrate (CAK assay) and

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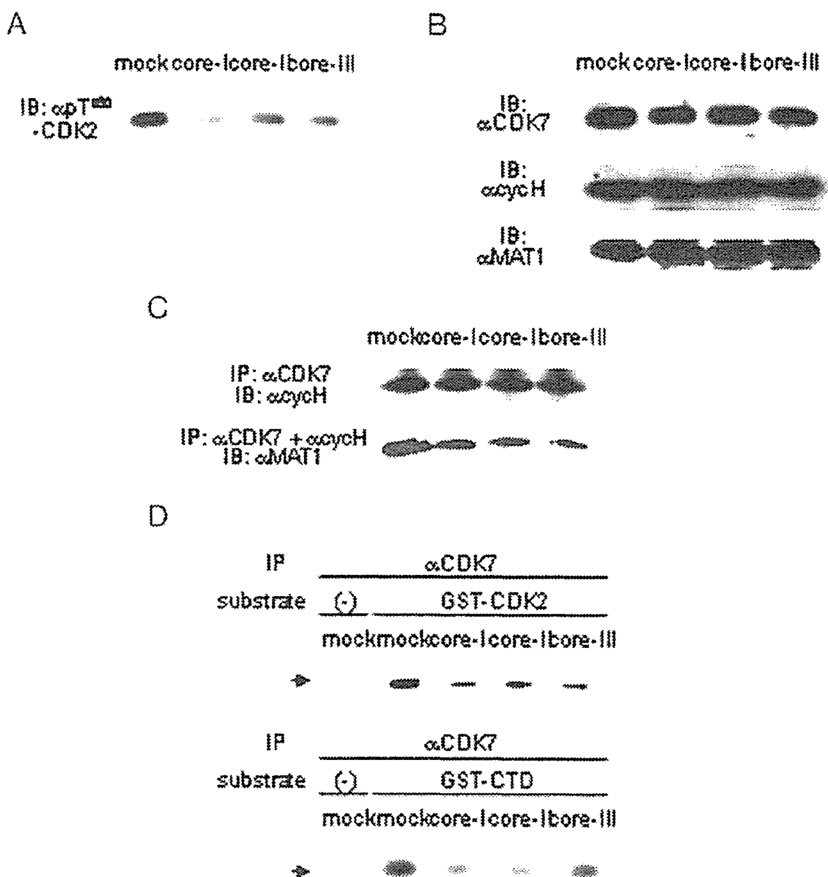

FIG. 4. **Expression, assembly, and activity of CAK in CL2 mock and core cells.** *A*, cellular proteins from CL2 mock and core cells were blotted with an antibody to pT160-CDK2. *B*, the expression levels of CDK7, cycH, and MAT1 were examined in CL2 mock and core cells by Western blotting. *C*, the CDK7-cycH complex and the CDK7-cycH-MAT1 (ternary CAK) complex were detected in CL2 mock and core cells by the IP/Western blot analysis. *D*, cellular proteins from CL2 mock and core cells were precipitated with a CDK7 antibody, and the precipitate was used for the kinase reaction with the GST-CDK2 fusion protein (for the CAK assay, *upper panel*) or the GST-CTD fusion protein (for the CTD kinase assay, *lower panel*) as a substrate. *IB*, immunoblot.

the GST-CTD substrate (CTD kinase assay) were further carried out (Fig. 4*D*). Both CAK and CTD kinase activities were suppressed in the CL2 core cells, compared with the mock cells. These findings indicate that persistent expression of HCV core may inhibit the ternary CAK complex formation, resulting in suppression of CAK activity. We also examined CDC25A expression by Western blotting and the tyrosine-phosphorylated CDK2 level using the IP reaction with a CDK2 antibody, followed by immunoblotting with a phosphorylated tyrosine antibody pY20 in CL2 mock and core cells. The expression level of CDC25A was not different between these cells. The tyrosine-phosphorylated CDK2 was below the detection limit in both CL2 mock and core cells (data not shown). According to these results, HCV core protein may not affect dephosphorylation of CDKs, although the CDC25A activity was not directly assessed in this study.

HCV Core Protein Directly Dissociates MAT1 from CAK Complex and Suppresses CAK Activity—As the next step, the direct effect of HCV core on CAK assembly and activity was investigated *in vitro*. Total cellular proteins from murine CL2 and human HepG2 cells were immunoprecipitated with a CDK7 antibody, followed by the binding reaction of the *in vitro* synthesized HCV core protein (aa 1–191) with the IP product. The *in vitro* translation product from the empty plasmid pCMVtag2B was also used as a negative control. The eluted product was then subjected to immunoblots using CDK7, cycH, and MAT1 antibodies and the CAK assay. As shown in Fig. 5*A*, the addition of the HCV core did not affect the levels of bound CDK7 and cycH but decreased the bound MAT1 level in both cases using CL2 and HepG2 cellular proteins. The CAK activity was also suppressed by the addition of HCV core (Fig. 5*B*). In these assays, the HCV core was detected very faintly in the eluted product by Western blotting (data not shown), indicating that the inhibitory effect on CAK assembly and activity may not have been due to an excess amount of HCV core. Furthermore, when the *in vitro* translated hepatitis B virus surface protein was used for this assay instead of the HCV core, the CAK activity was not suppressed by the addition of this protein (data not shown). Our findings strongly suggest that HCV core may dissociate MAT1 from the CAK complex through possible direct interaction with the particular CAK component, resulting in the suppression of CAK activity.

HCV Core Protein Interacts with CDK7—The direct binding of HCV core to CAK was further studied. The total cellular protein from the CL2 core-I cells was immunoprecipitated with a CDK7, cycH, or MAT1 antibody, and each precipitate was blotted with an antibody to the HCV core (Fig. 6*A*). In all cases using antibodies to CDK7, cycH, and MAT1 on the IP reaction, the three CAK components were precipitated efficiently. Nevertheless, HCV core was detected in the precipitates using CDK7 and cycH antibodies but not in that using a MAT1 antibody. This indicates that the HCV core may be directed against either CDK7 or cycH and that the interaction of the HCV core with the CDK7-cycH complex may induce dissociation of MAT1, as suggested above. To clarify the direct target molecule of HCV core, the *in vitro* GST fusion protein binding assay was carried out. The truncated HCV core protein (aa 1–122) fused to GST was used for this assay, because the full length of the HCV core fusion protein could not be obtained because of poor solubility. Total cellular proteins from murine CL2 and human HepG2 cells were incubated with the purified truncated HCV core protein, and the bound complex was checked by Western blotting using an antibody to CDK7 or cycH. The bound complex included the detectable level of

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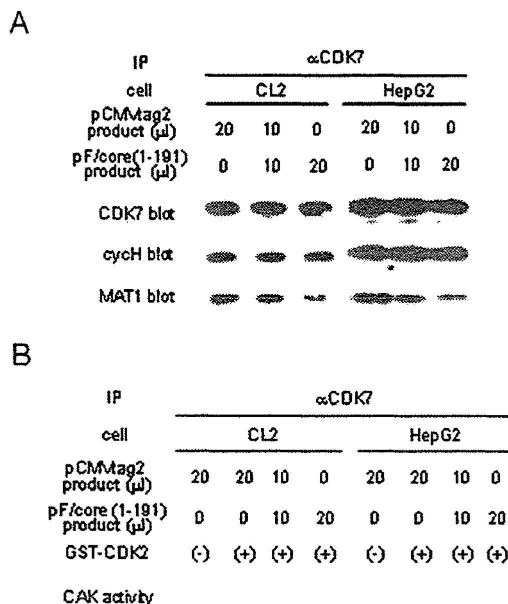

FIG. 5. **Direct effect on CAK assembly and activity by HCV core.** *A*, cellular proteins from CL2 (*left panel*) and HepG2 (*right panel*) cells were precipitated with a CDK7 antibody. The *in vitro* translation products containing various amounts of HCV core were added to the IP product, followed by the binding reaction. The product was then blotted with antibodies to CDK7, cycH, and MAT1. *B*, cellular proteins from CL2 (*left panel*) and HepG2 (*right panel*) cells were precipitated with a CDK7 antibody, and the precipitate was subjected to the binding reaction with the *in vitro* translation product containing the various amounts of HCV core. Then the sample was used for the CAK assay, as shown in Fig. 4*D*.

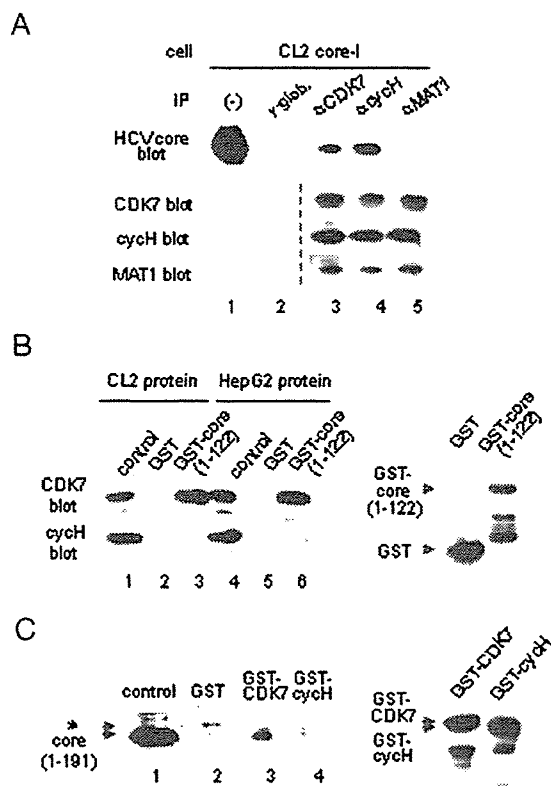

FIG. 6. **Binding of HCV core to CAK.** *A*, total cellular protein from CL2 core-I cells was precipitated with a rabbit nonspecific γ-globulin (negative control, *lane 2*), and antibodies to CDK7 (*lane 3*), cycH (*lane 4*), and MAT1 (*lane 5*), and the precipitates were blotted with antibodies to HCV core, CDK7, cycH, and MAT1. *Lane 1*, 5% of the cellular protein was used for Western blotting. *B*, *left panel*, GST alone (*lanes 2* and *5*) or the truncated HCV core protein (aa 1–122) fused to GST (*lanes 3* and *6*) was bound to the affinity resin and incubated with cellular proteins from CL2 (*lanes 2* and *3*) and HepG2 (*lanes 5* and *6*) cells. The bound protein complex was blotted with antibodies to CDK7 and cycH. *Lanes 1* and *4*, 10% of the cellular proteins from CL2 (*lane 1*) and HepG2 (*lane 4*) cells were used for Western blotting. *Right panel*, small portions of the products identical to *lanes 2* and *3* of the *left panel* were blotted with an antibody to GST. *C*, *left panel*, GST alone (*lane 2*), human CDK7 fused to GST (*lane 3*), or human cycH fused to GST (*lane 4*) was bound to the affinity resin and incubated with the *in vitro* translated full length of the HCV core protein. The bound protein complex was blotted with an antibody to HCV core. *Lane 1*, 10% of the HCV core protein was used for Western blotting. The *asterisk* shows the band possibly arising because of a weak cross-reaction of the GST protein with the antibody (*lane 2*). *Right panel*, small portions of the products identical to *lanes 3* and *4* of the *left panel* were blotted with an antibody to GST.

CDK7, whereas cycH was not detected in both cases using CL2 and HepG2 cellular proteins (Fig. 6*B*). Furthermore, human CDK7 and cycH fused to GST were synthesized and used for the binding reaction together with the *in vitro* translated full length of the HCV core protein (aa 1–191). HCV core was detected in the bound complex with GST-CDK7 but not in that with GST-cycH (Fig. 6*C*). Taken together, the HCV core protein could bind directly to both murine and human CDK7 proteins, whereas its binding to cycH may be indirect. Also, the region within aa 1–122 of HCV core was important for interaction with CDK7.

HCV Core Protein Does Not Influence Intracellular Localization of CAK—Finally, we investigated whether HCV core would affect the nuclear localization of CAK components. When the expression levels of CDK7, cycH, and MAT1 in the nuclear fraction were examined (Fig. 7*A*), they were not different between CL2 mock and core cells. We also investigated the intracellular localization of HCV core and CDK7 proteins in the CL2 core-I cells by immunostaining. As shown in Fig. 7*B*, the HCV core was expressed mainly in the cytoplasm but was also detected in the nucleus. On the other hand, CDK7 was expressed strongly in the nucleus, although the signal was seen sparsely in the cytoplasm. For the colocalization of both proteins, the HCV core was colocalized with a portion of CDK7 generally in the nucleus. Thus, the HCV core did not influence the nuclear translocation of CAK. The inhibitory effect of the HCV core on CAK may be exerted by its direct interaction with CDK7 in the nucleus.

DISCUSSION

In the present study, we aimed to elucidate the biological properties of the HCV core protein toward cell cycle progression and cell cycle-related molecules in the host cell. The HCV core-expressing stable transfectant, CL2 core, which was established from a murine normal liver-derived cell line (15, 41), was used for this purpose, because cells constitutively expressing the HCV core protein could not be isolated from the human hepatoma-derived cell lines Huh-7 (37), HepG2, and Hep3B (38). These CL2 core cells were shown to possess an only ~2–5-fold higher expression level of HCV core compared with that in the HCV-infected noncancerous liver tissue obtained from a patient with hepatocellular carcinoma who underwent partial hepatectomy (41). The HCV core has been shown to be immunohistochemically detected in a small portion of the hepatocytes of the HCV-infected liver specimen from chronic hepatitis C patients (48), suggesting that, unlike the cultured cells with forced expression of HCV core, the expression level of HCV core may differ among hepatocytes in the HCV-infected liver tissue. According to these, our CL2 core cells were speculated to express the physiological level of HCV core at least in a single cell.

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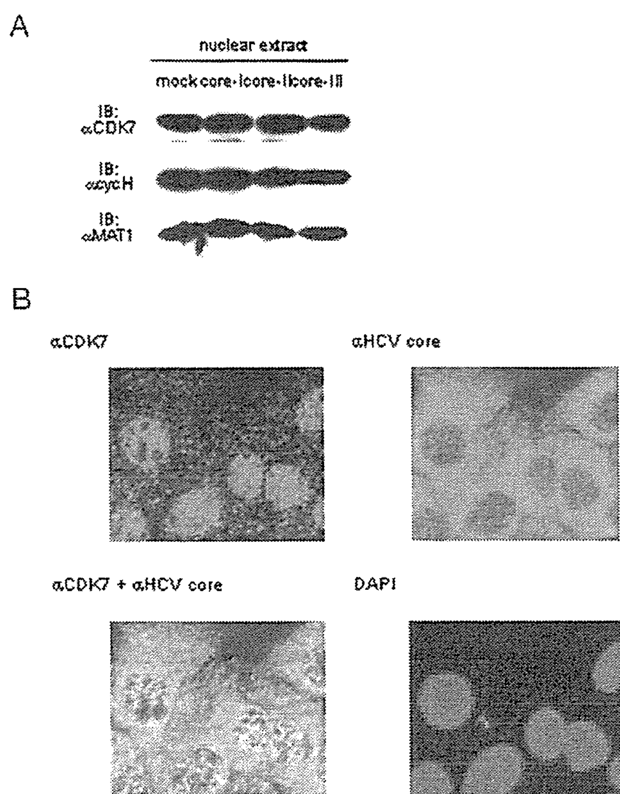

FIG. 7. **Intracellular localization of CAK in CL2 mock and core cells.** *A*, the nuclear fraction was obtained from CL2 mock and core cells, and the CDK7, cycH, and MAT1 proteins were detected by Western blotting. *B*, the CL2 core-I cells were double-stained with the mouse monoclonal HCV core antibody and the rabbit polyclonal CDK7 antibody and analyzed by the confocal microscopic analysis. Images recorded in *green* (HCV core) and *red* (CDK7) channels are shown separately in the *upper panels*, and a composite image is shown in the *left lower panel*. The *right lower panel* shows the nuclear staining with 4′,6-diamidino-2′-phenylindole-dihydrochrolide (*DAPI*). *IB*, immunoblot.

When the cell growth curve and the cell cycle profile were compared between CL2 mock and core cells, expression of HCV core impaired cell growth and the G_1/S transition in the cell cycle. It was also shown that persistent expression of HCV core suppressed CDK4 and CDK2 activities and subsequently inhibited the pRB phosphorylation and E2F-mediated transcription. Thus, HCV core-mediated inhibition of cell cycle progression was based on decreased CDK4 and CDK2 activities. We next carried out an extensive investigation on the factors modulating the CDK activity, which included the expression and complex formation of CDKs and cyclins, the expression levels of various CDK inhibitors, and the phosphorylation/dephosphorylation status of CDKs. It was found that the decrease of CDK2 phosphorylation caused by suppression of CAK activity was only a factor, which was identified as a possible cause of HCV core-mediated inhibition of CDK activity.

In the CL2 core cells, the expression levels of CAK components, CDK7, cycH, and MAT1 were not decreased, but the MAT1 level bound to the CDK7-cycH complex was suppressed, compared with the mock cells. Both CAK and CTD kinase activities were also lower in the CL2 core cells than in the mock cells. To investigate whether HCV core would directly modify the CAK activity, the binding reaction of the anti-CDK7 IP product with the *in vitro* synthesized HCV core protein was carried out using both murine CL2 and human HepG2 cellular proteins. It is noteworthy that the addition of the HCV core induced the release of MAT1 from the CAK complex and led to suppression of the CAK activity in the cases of both cellular proteins. These results strongly imply that the HCV core may directly interact with CAK and act as a suppressor of CAK by disrupting the ternary CAK complex in murine and possibly human cells.

The direct interaction of the HCV core with each of the CAK components was further examined. For *in vivo* interaction of HCV core with CAK in the CL2 core-I cells, as examined by the IP/Western blot analysis, it was revealed that the HCV core targeted either CDK7 or cycH. This suggests the existence of the HCV core-CDK7-cycH complex in cells. However, when the *in vitro* binding assay was carried out using the truncated HCV core protein fused to GST, CDK7, but not cycH, was included in the bound complex in both cases using CL2 and HepG2 cellular proteins. These discrepant results may have been due to the different conditions of the two assays. Otherwise, unlike the full length of the HCV core protein, the truncated HCV core protein would disrupt the complex formation of CDK7 and cycH. In either case, our finding indicates that the HCV core may directly bind to both murine and human CDK7 proteins. The result of another binding assay supports this, because the full length of HCV core protein could bind to GST-CDK7 but not to GST-cycH.

As for the intracellular localization of CAK, expression of the HCV core did not affect the nuclear translocation of the CAK components. In the immunofluorescence analysis, the colocalization of HCV core with CDK7 was found to occur mainly in the nucleus. This indicates that only a small portion of the HCV core, which is translocated to the nucleus, may interact with CDK7. It has been demonstrated that the complete loss of CAK results in cell death caused by the inability of cell cycle progression in the previous report using the cultured $mat1^{-/-}$ blastocytes (49). Our CL2 core cells may be able to survive because of incomplete suppression of CAK by the HCV core. According to this, it would be reasonable that only a portion of the HCV core protein is involved in the direct inhibitory effect on CAK.

It has been suggested by a few investigators that the HCV core may considerably affect the p53/$p21^{CIP1}$ status. It has been reported that the HCV core binds to both p53 and $p21^{CIP1}$ (21, 22) and impairs the cell cycle regulation in association with the increased expression of $p21^{CIP1}$ (22, 35). As for the p53 status in the CL2 cells used for this study, it has been shown that the CL2 cells possess the wild-type sequence of p53 gene and do not lose its expression (50). Therefore, the differences in the p53/$p21^{CIP1}$ status between CL2 mock and core cells were also examined in this study. In contrast to these previous reports, persistent expression of HCV core did not substantially affect the expression levels of p53 and $p21^{CIP1}$. Thus, HCV core-mediated suppression of cell cycle progression was not found to be responsible for modification of the p53/$p21^{CIP1}$ status in our CL2 cells. Such conflicting results may be due to the different expression level of the HCV core or different kinds of cultured cells.

CAK components are known to also be parts of the general transcription factor TFIIH and play an essential role not only in the cell cycle progression but also in transcription. TFIIH is composed of nine polypeptides, XPB, XPD, p62, p54, p44, p34, and three CAK components, CDK7, cycH, and MAT1 (51, 52). Biochemical analysis has revealed that CAK exists in three distinct forms in cells. The major form is a "free" ternary CAK complex, but CAK is also present as a CAK-XPD complex and the nine-subunit "holo" TFIIH (51). CAK phosphorylates the CTD of the largest subunit of RNA polymerase II (53), and phosphorylation of the CTD is believed to initiate promoter clearance and transcription elongation. In this study, the HCV core was found to suppress the CTD kinase activity by CAK,

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suggesting that the HCV core may affect basal transcription. Furthermore, TFIIH is an essential factor for nucleotide excision DNA repair in cells (54). It is speculated that the direct interaction of HCV core with CDK7 may disturb the formation of complete holo TFIIH complex, as well as the ternary CAK complex, leading to modification of TFIIH functions, such as transcription and nucleotide excision DNA repair. Further experimental evidence should lead to clarification of this.

Alterations between the CL2 mock and core cells may have been due to the artificial effects during establishment of the stable transfectant. To exclude this possibility, we conducted the cell-free assay using the anti-CDK7 IP product and the *in vitro* translated HCV core protein. As a result, the effects of HCV core on CAK assembly and activity in this assay were very similar to those observed in the assay using CL2 mock and core cells (Figs. 4 and 5). In addition, a difference in the E2F-mediated transcription activity between the CL2 mock and core cells was also confirmed in the cotransfection experiment using the CL2 cells (Fig. 2*A*). These results support that the phenotypic changes between the CL2 mock and core cells observed in this study should have been caused actually by persistent expression of the HCV core protein.

Our results conclusively showed that CAK is a novel target of the HCV core protein in the host cell. HCV core can directly bind to CDK7, and its binding causes dissociation of MAT1 from the CDK7-cycH complex and disrupts the stable ternary CAK complex. Such HCV core-mediated inhibition of CAK may prevent the activities of all cell cycle-related CDKs, including CDK2. This may be the molecular basis of HCV core-mediated suppression of cell cycle progression. It has recently been reported that a knock-out of the *mat1* gene led to embryonic lethality in mice because of the destabilization of CDK7 and cycH and the inability of cell cycle progression (49). Thus, CAK has been shown to be an indispensable factor to achieve cell cycle progression *in vivo*. According to this, it may be of great biological significance that the HCV core protein functions as an extrinsic suppressor of CAK in the host cell. In chronic HCV infection, the disease stage progresses with time accompanied by repeated liver cell injury and regeneration. The inhibitory effect on cell cycle progression caused by persistent expression of HCV core would strikingly affect this process in the pathogenesis of HCV-related liver diseases.

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Pseudotype hepatitis C virus enters immature myeloid dendritic cells through the interaction with lectin

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Abstract

Dendritic cells (DC) are the most potent antigen-presenting cells that regulate immune responses. One of the mechanisms for hepatitis C virus (HCV) persistence is the ability of HCV to suppress DC function. Direct HCV infection to blood DC has been implicated for DC dysfunction. To clarify the susceptibility of each DC subset to HCV, we used pseudotype vesicular stomatitis virus (VSV) coated with chimeric HCV envelope glycoproteins (E1 and E2). We demonstrate that pseudotype VSV enters myeloid DC (MDC) but not plasmacytoid DC (PDC). The highest efficiency of pseudotype VSV entry to MDC was observed when MDC were cultured with GM-CSF. Such efficiency decreased when MDC are matured with the treatment of IL-4, CpG oligodeoxynucleotide, or CD40 ligand. Mannan inhibited pseudotype VSV entry to MDC, but Ca^{2+} chelators failed to do so. These results show that pseudotype VSV possessing HCV-E1 and E2 enters immature MDC through the interaction with lectins in a Ca^{2+}-independent manner.
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Keywords: Hepatitis C virus; Envelope proteins; Pseudotype virus; Dendritic cells; Lectin

Introduction

Hepatitis C virus (HCV), a single-stranded plus-sense RNA virus belonging to the fraviviridae family (Miller and Purcell, 1990), causes persistent infection in more than 70% of infected patients. The most important feature of HCV persistence is the potential for liver disease progression from mild hepatitis to liver cirrhosis and hepatocellular carcinoma (HCC) (Alter et al., 1989). Chronic HCV infection is a serious health problem because the total number of HCV-positive HCC patients is growing worldwide. One of the mechanisms for HCV persistence is the ability of HCV to escape from the host cellular immune response (Farci et al., 1992; Weiner et al., 1995). Cumulative studies show that functional impairment of immunocompetent cells is found in patients with chronic HCV infection (Corado et al., 1997; Wedemeyer et al., 2002), suggesting that HCV has various arms for suppressing the immune response.

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) that regulate various immune responses (Banchereau and Steinman, 1998; Hart, 1997). Blood DC mainly consist of two subsets, that is, myeloid and plasmacytoid DC (Liu, 2001). Myeloid DC (MDC) are characterized by their potent immunostimulatory properties for both primary and secondary T-cell responses against virus. Plasmacytoid DC (PDC), previously known as interferon (IFN)-producing cells, produce a large amount of type I IFN upon virus infection (Liu, 2001). However, some viruses such as

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measles virus or HIV have been shown to infect monocyte-derived DC (MoDC) or PDC and subsequently inhibit their immunostimulatory functions (Grosjean et al., 1997; Patterson et al., 2001; Schnorr et al., 1997). Previous studies including our own have shown that MoDC from patients with chronic HCV infection are functionally impaired (Bain et al., 2001; Kanto et al., 1999). In addition, our recent investigation revealed that the function of both types of blood DC is suppressed as well in HCV-infected patients (Kanto T. et al., unpublished data). These results led us to hypothesize that HCV infection to DC is one of the mechanism for DC dysfunction in chronic hepatitis C patients.

The existence of the HCV genome in blood cells including DC has been shown in several studies by means of reverse transcription (RT)-PCR (Bain et al., 2001; Lerat et al., 1996, 1998; Navas et al., 2002). The detection of the positive strand of HCV-RNA does not enable to define whether HCV enters cells or only adheres to their surface. Alternatively, the negative strand of HCV-RNA has been used as a surrogate marker of HCV replication (Navas et al., 2002). Recently, Matsuura et al. (2001) established the pseudotype vesicular stomatitis virus (VSV) having chimeric HCV E1 and E2 protein as an envelope (VSV-E1E2). Because it has a green fluorescent protein (GFP) reporter gene in its genome, the infected cells can be viewed under fluorescence. Using this system, we tried to clarify the susceptibility of each DC to HCV. Consequently, we demonstrate that MDC is susceptible to VSV-E1E2 but PDC is not. Furthermore, we showed that the lectin-containing molecules on MDC are critically involved in VSV-E1E2 entry. Our study provides useful information for the exploration of target molecules that efficiently block HCV entry to DC.

Results

Immature MDC are susceptible to VSV-E1E2

We inoculated pseudotype VSV on various cells separated from PBMC or cord blood. Because no positive fluorescence was obtained from CD4 T cells, CD8 T cells, B cells, NK cells, and fresh PDC inoculated with VSV ΔG-G which is complemented with the VSV G protein, the susceptibility of these cells to VSV-E1E2 could not be estimated. In contrast, VSV ΔG-G entered fresh MDC, monocytes, and CD34$^+$ hematopoietic precursor cells on the day of separation, whereas VSV-E1E2 and VSV ΔG did not. Thus, fresh MDC as well as DC precursors are not susceptible to VSV-E1E2 (Fig. 1).

To examine the influence of differentiation or maturation of DC on the susceptibility to the pseudotype VSV, MDC were cultured in the presence of GM-CSF with or without IL-4. Phenotypic analysis revealed that day 4 MDC cultured with GM-CSF and IL-4 had higher expression of CD1a and CD86 than those cultured with GM-CSF only (Fig. 2), showing the role of IL-4 in DC maturation. No significant difference was observed in the expression of CD11c, CD40, CD80, CD83, and HLA-DR between these MDC (Fig. 2). On day 4 of culture in the presence of IL-3, PDC showed higher expressions of CD40, CD80, CD83, and CD86 when compared to the day of separation (data not shown).

After the inoculation with VSV ΔG-G, positive signals were obtained from day 4 MDC and day 4 PDC, regardless of the difference of cytokines used (Figs. 3A and B). No significant signals were detected from day 4 MDC and day 4 PDC inoculated with VSV ΔG. With respect to VSV-E1E2, GFP$^+$ cells were observed in day 4 MDC but not in day 4 PDC (Figs. 3A and B). In

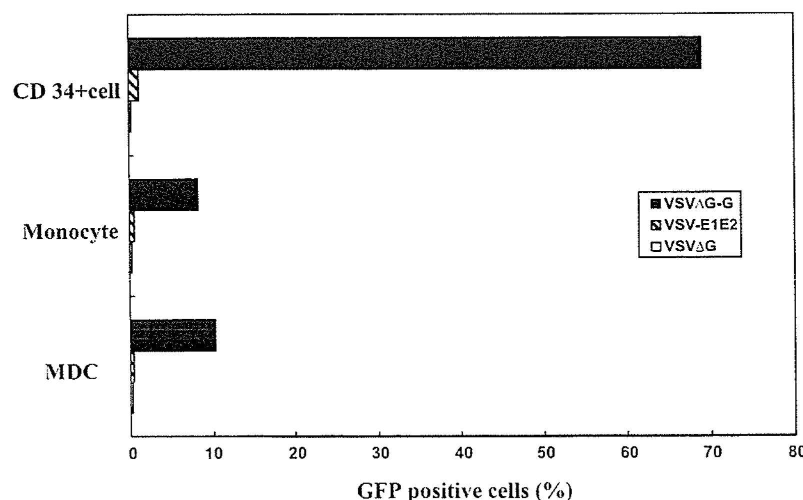

Fig. 1. Freshly isolated DC are not susceptible to VSV-E1E2. Freshly isolated CD34$^+$ hematopoietic precursor cells, monocytes, or MDC were inoculated with pseudotype VSVs and the percentages of GFP$^+$ cells were determined by flow cytometry. Representative results from three experiments are shown.

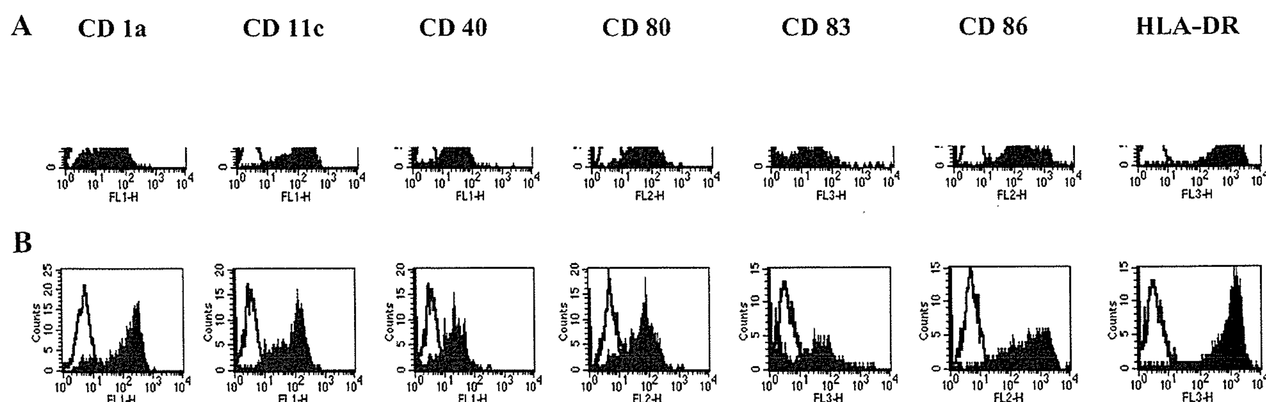

Fig. 2. Phenotypes of MDC cultured with GM-CSF or GM-CSF and IL-4. Flow cytometric analyses of surface molecules on day 4 MDC, obtained from healthy volunteers, cultured with GM-CSF (A) or with GM-CSF and IL-4 (B). Representative results from three subjects are shown. Open histograms represent the results with isotype Ab and filled ones represent those with relevant Abs. Fluorescence intensity is shown in the *x*-axis and the number of cells is shown in the *y*-axis.

comparison of the culture conditions for MDC, higher percentage of GFP$^+$ cells was observed in day 4 MDC cultured with only GM-CSF than those with GM-CSF and IL-4 (Fig. 3B).

To confirm the reliability of the pseudotype VSV system in the assessment of E1E2-mediated virus entry, we quantified HCV-RNA in day 4 cultured MDC or PDC after inoculation of a window-period serum from a hepatitis C patient. Among the cells examined, the highest HCV RNA titer was detected in day 4 MDC cultured with GM-CSF (Fig. 3C), which was compatible with the results obtained with the pseudotype VSV. In contrast with the results of VSV-E1E2 inoculation, low-level HCV RNA were detected by quantitative RT-PCR in PDC inoculated with authentic HCV (Fig. 3C). To further investigate whether HCV replicates in each DC subset after HCV inoculation, we performed strand-specific RT-PCR for the detection of negative-strand HCV-RNA as a surrogate marker of HCV replication. Positive strand of HCV-RNA was detected both in MDC cultured with GM-CSF and PDC with IL-3, whereas negative strand was detected in GM-CSF-MDC but not in IL-3-PDC (Fig. 3D). These results suggest that HCV replicates in GM-CSF-MDC but not in IL-3-PDC. Therefore, the data with the pseudotype VSV system correctly reflect the susceptibility of cells to authentic HCV.

Maturation stimuli protect MDC from VSV-E1E2

Based on the findings described above, we hypothesized that the more MDC mature, the less susceptible they are to VSV-E1E2. To find the substances protecting DC from HCV infection, we treated MDC with various maturation factors for the inoculation study. In MDC cultured with GM-CSF, the addition of IL-4, CpG oligodeoxynucleotide (ODN) 2006, or CD40 ligand (CD 40L) to the culture significantly reduced the percentage of GFP$^+$ cells with VSV-E1E2 without influencing their susceptibility to VSV ΔG-G (Fig. 4). On the other hand, IFN-α, polyI:C, TNF-α, and lipopolysaccharide (LPS) reduced the percentage of GFP$^+$ cells with both VSV-E1E2 and VSV ΔG-G (Fig. 4). Phenotypic analysis revealed that IL-4 up-regulated the expression of CD1a and CD86 on MDC cultured with GM-CSF (Fig. 2). CpG ODN or CD40L also up-regulated the expression of CD1a, CD83, and CD86 on MDC cultured with GM-CSF (data not shown). Therefore, immature DC lose their susceptibility to VSV-E1E2 as they develop to be more mature state.

Lectin on DC is involved in VSV-E1E2 entry to DC

The C-type lectins expressed on DC are reported to interact with various viruses as well as microbial agents (Geijtenbeek et al., 2000; Tailleux et al., 2003). These studies led us to consider the involvement of lectins in VSV-E1E2 entry to DC. Thus, we first used mannan to examine whether it inhibits VSV-E1E2 entry to MDC. The pretreatment of MDC with mannan reduced the percentage of GFP$^+$ cells with VSV-E1E2 in a dose-dependent manner without having any impact on VSV ΔG-G entry (Fig. 5A). Such an inhibitory effect of mannan was confirmed with MDC inoculated with authentic HCV (data not shown). A D-mannose-specific lectin, methyl α-D-mannopyranoside (Kaku et al., 1991), also inhibited VSV-E1E2 entry to MDC in a dose-dependent fashion at concentrations from 10 to 40 μg/ml (data not shown). In contrast, galactose had no effect on the infection with either VSV-E1E2 or VSV ΔG-G in MDC (Fig. 5A). Interestingly, EDTA did not reduce the infectivity of VSV-E1E2, whereas it completely abolished that of VSV ΔG-G (Fig. 5A). These data demonstrate that mannose-type carbohydrate is involved in the interaction of DC with VSV-E1E2 in a Ca^{2+}-independent manner. The treatment of MDC with antihuman DC-SIGN Ab,

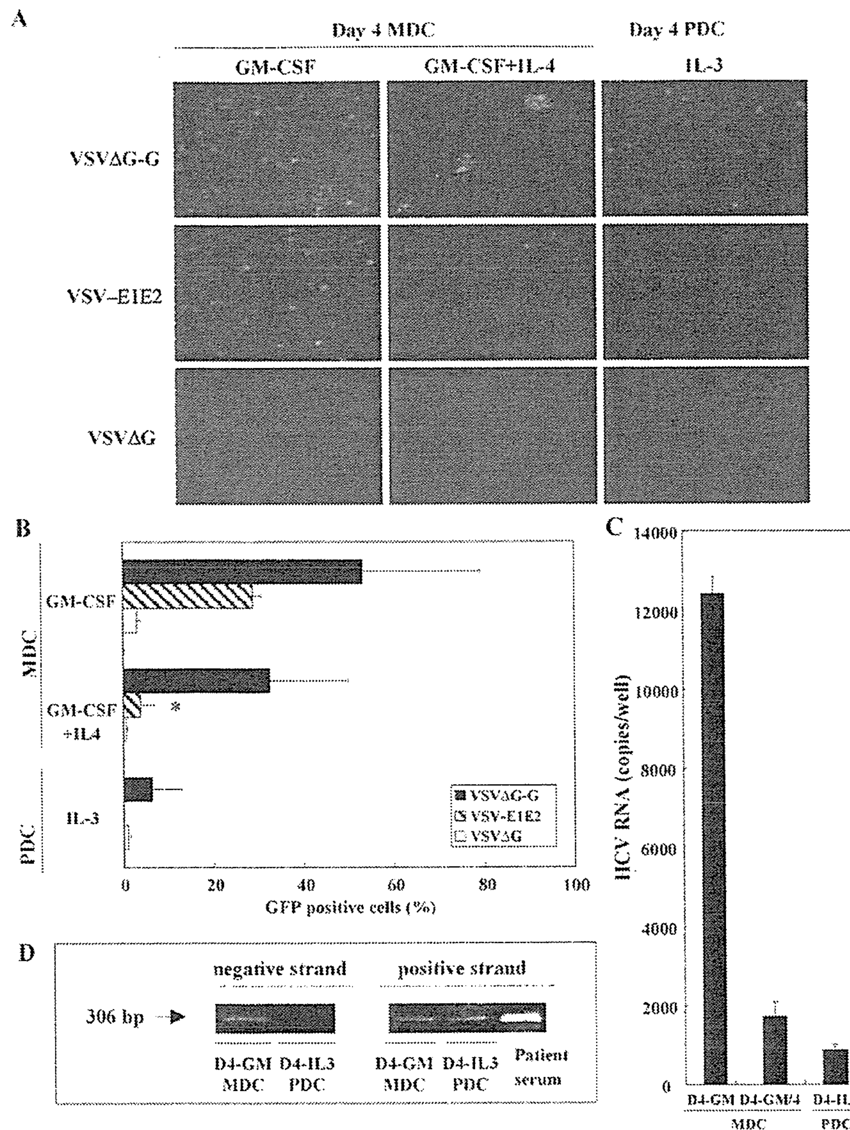

Fig. 3. Myeloid DC cultured with GM-CSF are susceptible to VSV-E1E2 or authentic HCV from patient serum. Day 4 MDC cultured with GM-CSF or with GM-CSF and IL-4 or day 4 PDC cultured with IL-3 were inoculated with VSV ΔG, VSV ΔG-G, or VSV-E1E2. They were viewed under fluorescence microscopy (A) and the percentages of GFP^+ cells were analyzed by flow cytometric analysis (B). The results of fluorescence microscopy are the representative ones from three subjects. The results of flow cytometric analysis are expressed as the mean + SD from three representative experiments. $*P < 0.001$ vs. VSV-E1E2-inoculated day 4 MDC cultured with GM-CSF. (C) Quantitative analysis of HCV RNA in DC inoculated with HCV-positive patient serum was performed as described in Materials and method. D4-GM or D4-GM/4 represents MDC cultured with GM-CSF or GM-CSF and IL-4 for 4 days. D4-IL 3 represents PDC cultured with IL-3 for 4 days. The results are expressed as the mean + SD of triplicate wells from three representative experiments. (D) The detection of positive and negative strand of HCV-RNA in DC inoculated with HCV-positive patient serum. Strand-specific RT-PCR was performed with samples from MDC, PDC, and patient serum used as inoculum, as described in Materials and method. D4-GM and D4-IL 3 represent as the same as above.

which is able to block the binding of DC-SIGN to ICAM-3 (Wu et al., 2002), did not inhibit the entry of either VSV-E1E2 or VSV ΔG-G (Fig. 5B). To see whether the expression of DC-SIGN on MDC parallels their susceptibility to VSV-E1E2, we compared the expression of DC-SIGN between MDC cultured with GM-CSF and those with a combination of GM-CSF and IL-4. The expression of DC-SIGN was higher on MDC cultured with GM-CSF and IL-4 than on those with GM-CSF (Fig. 5C), which is contrary to their susceptibility to VSV-E1E2. These results show that DC-SIGN is less likely to be involved in the VSV-E1E2 entry to MDC. Human hepatoblastoma cell line, HepG2, is one of the most sensitive cells to pseudotype VSV (Matsuura et al., 2001). To compare the machinery of VSV-E1E2 entry between MDC and HepG2, we inoculated it to mannan-treated HepG2. In contrast to MDC, mannan did not inhibit the VSV-E1E2 entry to HepG2 (Fig. 5D), suggest-