厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業

マイクロ RNA を標的とした新規抗 C 型肝炎ウイルス治療戦略の開発

平成２４年度　総括研究報告書

研究代表者　　渡士　幸一

平成２５（２０１３）年　３月

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I. 総括研究報告

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）

総括研究報告書

マイクロRNAを標的とした新規抗C型肝炎ウイルス治療戦略の開発

研究代表者　渡士　幸一　国立感染症研究所　ウイルス第二部　主任研究官

研究要旨：本研究では、マイクロRNA等宿主因子を標的とした新規治療法の開発を目的とし、 主に(1) miRNA阻害剤TPFによるHCV複製抑制作用メカニズムの解析、(2) TPF誘導体のmiRNA経路への効果、抗HCV作用の解析、(3) TPFとは異なるmiRNA阻害剤、抗HCV剤の探索、(4)(3)の化合物の抗HCV作用機構の解析　を行った。その結果、TPFはAGO2複合体へのmiR-122およびRNA helicase Aの会合を低下させることが観察され、またTPFは少なくとも我々の実験系ではHCV翻訳を阻害することが示された。またTPFとは別系統のmiRNA阻害剤、抗HCV剤をスクリーニングしたところ、coumermycinA1がmiRNA産生を阻害するものとして得られた。またこれ以外に16化合物を抗HCV効果をもつ化合物として同定した。これらの中にはHCV粒子形成、放出を阻害する化合物などが含まれていた。今後これらの化合物のmiRNA経路への影響および抗HCV作用機構を解析することにより、miRNA等宿主因子を標的とする新規抗HCV剤の開発を進めたい。

A. 研究目的

C型肝炎ウイルス(HCV)感染者は本邦において100から200万人存在すると推定されている。HCV治療としては現在まで主にペグインターフェロンとリバビリンの併用療法が用いられていたが、その著効率は約50%にとどまることから、新たな抗HCV療法が求められている。特に長期投与に伴う薬剤耐性ウイルス出現を克服するためには、宿主因子を標的とした治療薬開発が重要である。本研究ではマイクロRNA(miRNA)等の宿主因子を標的とした新規治療法の開発を目指す。miRNAは多様な宿主遺伝子発現を調節する小分子RNAであり、その発現および機能異常はがんや分化異常などさまざまな疾患を引き起こすことが知られる。肝臓に高発現するmiR-122はHCVの宿主細胞域を規定する複製補因子として認知されており、細胞内でのHCVゲノム複製を強く促進する。これまでに研究代表者はさまざまなmiRNA種の遺伝子サイレンシング経路を低分子化合物により阻害できることを初めて示した。本研究ではまずmiRNAによるHCV複製機構の解析および抗HCV剤開発を目的として、このmiRNA阻害剤TPFを用いる。またこれとは別のmiRNA阻害剤ならびに抗HCV化合物を探索する。得られた化合物のmiRNA阻害および抗HCV効果の分子メカニズムの解明をおこなうことにより、抗HCV剤の創薬標的の同定を目指す。

B. 研究方法

具体的な実験・解析方法は以下の通りである。

(1) <u>miRNA阻害剤によるHCV複製抑制作用メカニズムの解析</u>

AGO2複合体内miR-122量およびRNA helicase A (RHA)は、TPF処理、未処理のHuh-7細胞を溶解後抗AGO2抗体で免疫沈降し、沈降産物内のmiR-122およびRHAをreal time RT-PCR法およびイムノブロット法で検出した。

また細胞内総miR-122量およびRHAは、細胞溶解液を免疫沈降せずにreal time RT-PCR法およびイムノブロット法で解析した。

(2) さらに抗HCV効果の高いmiRNA阻害剤の探索

TPF誘導体としてSigma-Aldrich等より購入した15種類の化合物を用いた。サイレンシング活性は、ホタルルシフェラーゼ(Fluc)、ウミシイタケルシフェラーゼ(Rluc)およびFlucに対するshRNA発現プラスミドをトランスフェクションした細胞を用いてレポーターアッセイを行い、FlucをRlucにより割った値の相対値により定量した。またHCV RNA複製は、Flucを有するHCVレプリコンを用いたレポーターアッセイにより測定した。

(3) TPFとは別系統のmiRNA阻害剤の同定

Huh-7細胞にさまざまな化合物を処理した後の細胞内総miR-122量をreal time RT-PCR法により定量した。細胞毒性はMTTアッセイにより調べた。

(4) 新規抗HCV化合物の同定

Huh-7-25細胞にHCV JFH1 RNAをエレクトロポレーションで導入した。4時間後に各化合物を処理し、72時間後の培養上清に放出されたHCVの感染性を測定した。HCV感染性は、Huh-7.5.1細胞に再感染させた48時間後の細胞内HCV RNAをreal time RT-PCR法で定量することにより測定した。

(5) (4)で得られた化合物の抗HCV作用機構の解析

HCV生活環中の、吸着・侵入を含む初期過程はHCVシュード粒子(HCVpp)系により検討した。翻訳・RNA複製を含む中期過程はHCVレプリコン系により評価した。前者においては、Huh-7.5.1細胞に各化合物を1時間前処理した後にHCVppを4時間感染させ、72時間後にHCVpp感染によって発現されるルシフェラーゼ活性を測定した。後者のアッセイでは、HCVレプリコンRNAをHuh-7.5.1細胞にエレクトロポレーションで導入した4時間後に各化合物を処理し、さらに48時間後のレプリコンRNAから産生されるルシフェラーゼの活性を定量することにより、HCV翻訳・RNA複製を評価した。

(倫理面への配慮)

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

C. 研究結果

(1) miRNA阻害剤によるHCV複製抑制作用メカニズムの解析

1. TPFがargonaute 2複合体へ与える影響

TPFはHuh-7細胞内の総miR-122量には影響を与えなかったが、AGO2複合体内のmiR-122量を有意に減少させた。またAGO2複合体へのmiR-122の取り込みに関わるRNA helicase A (RHA)とAGO2との相互作用がTPF処理により解離することが観察された。一方AGO2複合体内miR-122量に影響を与えないTPF誘導体ACDは、RHAとAGO2の相互作用を変化させなかった。

2. TPFのHCV生活環内標的ステップの解析

TPF がHCV生活環内のどのステップを阻害するのかをHCVレプリコンを用いて解析した。その結果、TPFはHCV RNA安定性には大きな変化は与えなかったが、HCV翻訳およびそれに続くRNA複製を大きく抑制した。

(2) さらに抗HCV効果の高いmiRNA阻害剤の探索

miRNAサイレンシングおよびHCVゲノム複製への効果を、合計15種類のTPF誘導体について調べた。これらのうち1種類が強い、2種類が中程度もしくは弱いshRNA誘導サイレンシング抑制効果を有していた。強いサイレンシング抑制効果を持つ誘導体は、顕著なHCVゲノム複製抑制を示した。しかしながらTPF誘導体中、TPFよりも明らかに強い抗HCV効果を有する化合物は得られなかった。

(3) TPFとは別系統のmiRNA阻害剤の同定

TPFを含む合剤であるACFはマウス体内で速やかに代謝・排泄された。そのためTPFとは別

のmiRNA阻害剤および抗HCV化合物を同定する必要があると考え、Huh-7細胞内の総miR-122量を減少させる低分子化合物のスクリーニングをおこなった。その結果coumermycinA1が細胞内総miR-122量を低下させることが明らかとなった。

(4) 新規抗HCV化合物の同定

一方、HCV粒子産生培養系を用いて感染性HCV産生を抑制する低分子化合物をスクリーニングした。その結果、coumermycinA1の他に、さらに16化合物を感染性HCV産生を低下させるものとして同定した。

(5) (4)で得られた化合物の抗HCV作用機構の解析

得られた低分子化合物がHCV生活環中のどのステップを阻害するかを検討したところ、1化合物が吸着・侵入を含む初期過程、coumermycinA1を含む4化合物が翻訳・RNA複製といった中期過程を、また12化合物は粒子形成から放出に至る生活環後期過程を阻害することが示唆された。このうち生活環後期過程に影響を与えた化合物に着目したところ、粒子形成を低下させる化合物、また放出を阻害する化合物がそれぞれ存在することが示唆された。

D. 考察

(1) miRNA阻害剤によるHCV複製抑制作用メカニズムの解析

今回の結果より、TPFはAGO2複合体内のmiR-122量を減少させるが、それはAGO2とRHAの相互作用の解離を伴うことが判明した。このことより、TPFはRHAによるAGO2の機能制御を解除することによりmiR-122のAGO2複合体への取り込みを阻害する可能性、あるいはTPFはAGO2とmiR-122およびRHAの相互作用を同時に解除する可能性が考えられた。

(2) さらに抗HCV効果の高いmiRNA阻害剤の探索

今回試験した化合物の中ではTPF自体が最も抗HCV効果が高かった。そこでTPFを含む合剤であるACFをin vivo HCV感染モデルマウスおよびddYマウスに投与したが、この化合物はマウス血中から速やかに消失した。このことより、マウス血中で薬物動態がより好ましいTPF誘導体を同定するとともに、TPFとは異なる骨格をもつmiRNA阻害剤および抗HCV化合物を同定する必要があると考えられた。

(4) TPFとは別系統のmiRNA阻害剤、抗HCV化合物の同定

今回、Huh-7細胞を用いた低分子化合物のスクリーニングにより、coumermycinA1が細胞内総miR-122量を低下させることが示された。またこれはHCV翻訳・RNA複製ステップを阻害した。つまりcoumermycinA1はmiR-122産生低下を介してHCV翻訳あるいは複製を阻害する可能性が考えられた。今後coumermycinA1の作用メカニズムのさらなる解析および抗HCV効果のプロファイルを調べる。

またcoumermycinA1以外にHCV粒子産生を低下させる16化合物を新たに得た。これらはそれぞれHCV生活環すべてにわたる異なるステップに作用することが示唆されたので、これらの低分子化合物はHCV生活環分子メカニズムの全貌を明らかにするためのよいツールになりうると期待できる。これまでおこなった解析では、HCV粒子形成および放出を阻害する化合物において、その抗HCV作用に宿主酵素あるいは受容体の阻害が重要である可能性が示唆されている。これらのヒット化合物を用いてHCV生活環に必要なさまざまな宿主因子が明らかになるかもしれない。またその中から抗HCV剤の創薬標的を同定することを今後目指す。

E. 結論

miRNA阻害剤TPFはmiR-122とAGO2複合体の相互作用を低下させ、HCV翻訳を阻害することが示唆された。一方、今回coumermycinA1をmiR-122産生を低下させるものとして同定した。またこれとは別にさらに16化合物を抗HCV効果を有するものとして今回新たに得た。今後化合物のmiRNA経路への影響ならびに抗HCV分子メカニズムを解析することにより、宿主因子を標的とする新規抗HCV剤の取得を目指す。

F. 健康危険情報

特記事項なし。

G. 研究発表

1. 論文発表

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H. 知的所得権の所得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

II. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Weng L, Tian X, Gao Y, Watashi K, Shimotohno K, Wakita T, Kohara M, Toyoda T	Different mechanisms of hepatitis C virus RNA polymerase activation by cyclophilin A and B in vitro	Biochim Biophys Acta	1820	1886-1892	2012
Murayama A, Sugiyama N, Watashi K, Masaki T, Suzuki R, Aizaki H, Mizuochi T, Wakita T, Kato T	Japanese reference panel of blood specimens for evaluation of hepatitis C virus RNA and core antigen quantitative assays	J Clin Microbiol	50	1943-1949	2012
渡士幸一	HBV 培養細胞系による新規抗ウイルス化合物のスクリーニング	肝胆膵	65	611-617	2012
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III. 研究成果の刊行物・別刷

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Different mechanisms of hepatitis C virus RNA polymerase activation by cyclophilin A and B in vitro

Leiyun Weng [a], Xiao Tian [a], Yayi Gao [a], Koichi Watashi [b], Kunitada Shimotohno [c], Takaji Wakita [b], Michinori Kohara [d], Tetsuya Toyoda [a,d,e,*]

[a] Unit of Viral Genome Regulation, Institut Pasteur of Shanghai, Chinese Academy of Sciences, 411 Hefei Road, 200025 Shanghai, People's Republic of China
[b] Department of Virology II, National Institute of Health, 1-23-1 Toyama, Shinjuku, Tokyo 132-8640, Japan
[c] Chiba Institute of Technology, 2-17-1 Tsudamuna, Narashino, Chiba 275-0016, Japan
[d] Department of Microbiology and Cell Biology, The Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-Ku, Tokyo 156-8506, Japan
[e] Choju Medical Institute, Fukushimura Hospital, 19-14 Azanakayama, Noyori-cho, Toyohashi, Aichi 441-8124, Japan

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ABSTRACT

Background: Cyclophilins (CyPs) are cellular proteins that are essential to hepatitis C virus (HCV) replication. Since cyclosporine A was discovered to inhibit HCV infection, the CyP pathway contributing to HCV replication is a potential attractive stratagem for controlling HCV infection. Among them, CyPA is accepted to interact with HCV nonstructural protein (NS) 5A, although interaction of CyPB and NS5B, an RNA-dependent RNA polymerase (RdRp), was proposed first.
Methods: CyPA, CyPB, and HCV RdRp were expressed in bacteria and purified using combination column chromatography. HCV RdRp activity was analyzed in vitro with purified CyPA and CyPB.
Results: CyPA at a high concentration (50× higher than that of RdRp) but not at low concentration activated HCV RdRp. CyPB had an allosteric effect on genotype 1b RdRp activation. CyPB showed genotype specificity and activated genotype 1b and J6CF (2a) RdRps but not genotype 1a or JFH1 (2a) RdRps. CyPA activated RdRps of genotypes 1a, 1b, and 2a. CyPB may also support HCV genotype 1b replication within the infected cells, although its knockdown effect on HCV 1b replicon activity was controversial in earlier reports.
Conclusions: CyPA activated HCV RdRp at the early stages of transcription, including template RNA binding. CyPB also activated genotype 1b RdRp. However, their activation mechanisms are different.
General significance: These data suggest that both CyPA and CyPB are excellent targets for the treatment of HCV 1b, which shows the greatest resistance to interferon and ribavirin combination therapy.

1. Introduction

Hepatitis C virus (HCV[1]), which belongs to the *Flaviviridae* family, has a positive-strand RNA genome, and its replication is regulated by viral and cellular proteins [1]. The genome encodes a large precursor polyprotein that is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [2]. NS5B is an RNA-dependent RNA polymerase (RdRp) [3–5].

HCV frequently establishes a persistent infection that leads to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [6,7]. More than 170 million individuals worldwide are infected with HCV [8], and the challenge of developing HCV treatment continues. First, combination therapy with pegylated interferon α (Peg-IFNα) and ribavirin led to a sustained virological response (SVR) in approximately 55% of patients infected with any HCV genotype and 42–46% of patients with genotype 1 [9,10]. However, many patients could not tolerate the serious adverse effects. Triple therapy consisting of an NS3/NS4A protease inhibitor (boceprevir or telaprevir), Peg-IFN (α-2a or α-2b), and ribavirin was then introduced, and it has become the standard regimen for genotype 1 infection. SVR improved significantly (from 63% to 75%), and the treatment duration decreased from 12 to 6 months [11,12]. However, triple therapy is more toxic than combination therapy [13].

Nonimmunosuppressant cyclosporine A (CsA) analogues/CyP inhibitors such as DEBIO-025 (Alisporivir) [14], NIM811 [15], and SCY-635 [16] are also the most expected candidates for use as anti-HCV drugs because their resistance selection is rare compared with other direct-acting antiviral agents, and the HCV resistant to

Abbreviations: BSA, bovine serum albumin; CsA, cyclosporine A; CyP, cyclophilin; DTT, dithiothreitol; E, envelope; EDTA, ethylenediaminetetraacetic acid; GST, glutathione S-transferase; HCV, hepatitis C virus; NS, nonstructural protein; PPI, peptidyl prolyl *cis/trans*-isomerases; Peg-IFN, pegylated interferon-α; PMSF, phenylmethanesulfonylfluoride; RT-PCR, reverse transcription polymerase chain reaction; RdRp, RNA-dependent RNA polymerase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis; SVR, sustained virological response; ΔPPI, PPI knockout; wt, wild type

* Corresponding author at: Choju Medical Institute, Fukushimura Hospital, 19-4 Azanakayama, Noyori-cho, Toyohashi, Aichi 441-8124, Japan. Tel.: +81 532 46 7511; fax: +81 532 46 8940.
E-mail address: toyoda_tetsuya@yahoo.co.jp (T. Toyoda).

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CyP inhibitors acquired mutations that allowed for reduced dependence on CyPs [17,18].

CyP was originally discovered as a cellular factor with high affinity for CsA [19]. CyPs comprise a family of peptidyl prolyl *cis/trans*-isomerases (PPI) that catalyze the *cis–trans* interconversion of peptide bonds amino terminal to proline residues, facilitating protein conformation changes [20]. CyPs are potential antiviral targets because CyPA was found to play a critical role in human immunodeficiency virus-1 infection [21,22]. The role of human CyPs as cellular cofactors in HCV replication was first suggested upon discovery of the anti-HCV effect of CsA [23–26]. Although the completion of a binding assay and the mapping of resistance initially suggested that NS5B was a viral target for CsA [27–29], recent papers have pointed to CyPA and NS5A as the central virus–host interaction involved in HCV replication [30–36]. Despite this unfavorable evidence, we analyzed the effect of CyPA and CyPB on HCV RdRp of various genotypes in vitro and found differences in genotype specificity and the mechanism of HCV RdRp activation.

2. Materials and methods

2.1. Purification of HCV RdRp

HCV RNA RdRps with C-terminal 21 amino acid deletion of 1a (H77 and RMT), 1b (HCR6, NN, and Con1), and 2a (JFH1 and J6CF) were expressed in *E. coli* Rosetta/pLysS and purified as described previously [37–40]. The purified HCV RdRps (5 μM, >95% pure) were stocked in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 5% glycerol, and 1 mM phenylmethanesulfonylfluoride (PMSF) at −80 °C. The yield of HCV RdRps is approximately 1.7 mg from a 1-L bacterial culture. The purified HCV RdRps were as shown in Fig. S1 of Weng et al. [38]. The protein purities were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (SDS-PAGE), using ImageJ 1.46 (http://rsbweb.nih.gov/ij/).

2.2. Construction of CyP-expressing plasmids

Human CyPA and CyPB were cloned from total RNA extracted from 293T cells, using a reverse transcription-polymerase chain reaction (RT-PCR) kit (Takara, Dalian, China) as published previously [29]. After being digested with *Bam*HI and *Eco*RI, they were cloned into the same site of pGEX-6P-3 (GE Healthcare, Bucks, UK), resulting in pGEXCyPA and pGEXCyPB, respectively. CyPBΔPPI, the enzymatic inactive mutant of CyPB, was PCR cloned into pGEX-6P-3 from pCMV-CyPBΔPPIFL [29], resulting in pGEXCyPBΔPPI. CyPAΔPPI was produced by the introduction of the R55A and F60A mutations using a QuickChangeII Site-Directed Mutagenesis Kit (Stratagene, St. Clara, CA, USA) and primers (5′-GTTCCTGCTTTCACGCCATTATTCCAGGGG CCATGTGTCAGGGTG-3′ and 5′-CACCCTGACACATGGCCCCTGGAATAA TGGCGTGAAAGCAGGAAC-3′).

2.3. Purification of CyPs

E. coli Rosetta were transformed using pGEXCyPA, pGEXCyPAΔPPI, pGEXCyPB, and pGEXCyPBΔPPI. GST-tagged CyPA, CyPB, CyPAΔPPI, and CyPBΔPPI were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at 18 °C for 4 h. The bacteria were harvested and stocked at −20 °C. After thawing on ice, the bacteria were lysed in 4 packed cell volumes of phosphate-buffered saline, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. After being clarified by centrifugation at 10,000×*g* for 30 min at 4 °C and filtered through a 0.45-μm nitrocellulose filter, the extract was incubated with Glutathione Sepharose 4B (GE Healthcare) for 30 min at 4 °C. After the resin was washed with 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, the GST-CyP was eluted using 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM reduced glutathione, and 1 mM PMSF, followed by gel filtration through a Superdex 200 column (GE Healthcare) in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. The eluted GST-CyP were diluted to 50 mM NaCl and applied to a MonoQ (GE Healthcare) in 20 mM Tris–HCl (pH 9.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. GST-CyPB and GST-CyPBΔPPI were chromatographed using a continuous NaCl gradient of 50–1000 mM. The purified CyPs were stocked at −20 °C.

2.4. In vitro HCV transcription with CyPs

In vitro HCV transcription with CyPs was done as previously described [37–40]. Briefly, the indicated amounts of the CyPs were incubated in 50 mM Tris–HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM $MnCl_2$, 1 mM DTT, 0.5 mM GTP, 200 nM of a 184-nt in vitro transcribed model RNA template (SL12-1S), 100 U/mL of human placental RNase inhibitor, and 100 nM HCV RdRp at 29 °C for 30 min. After preincubation, RdRp was incubated for an additional 90 min with 50 μM ATP, 50 μM CTP, or 5 μM [α-^{32}P]UTP. The RNA products were analyzed using 6% PAGE containing 8 M urea after being purified by phenol/chloroform extraction and ethanol precipitation. The amount of RNA products was analyzed using Typhoon Trio (GE Healthcare).

2.5. RNA filter-binding assay with CyPA and CyPB

An RNA filter-binding assay with CyPA and CyPB was performed as previously described [37,38,40]. Briefly, [^{32}P]-SL12-1S was incubated in 25 μL of 50 mM Tris–HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM $MnCl_2$, 1 mM DTT, and 5 pmol of HCV RdRp with 375 pmol (75×) of CyPA and 25 pmol (5×) of CyPB at 29 °C for 30 min.

2.6. Chemicals and radioisotopes

[α-^{32}P]UTP (800 Ci/mmol, 40 mCi/mL) was purchased from PerkinElmer Life Sciences (Waltham, MA, USA). The nucleotides were purchased from GE Healthcare. The human placental RNase inhibitor T7 RNA polymerase and PrimeSTAR HS DNA polymerase were purchased from Takara. The bacteria were purchased from Novagen (Merck Chemicals, Darmstadt, Germany).

2.7. Statistical analysis

The statistical data were evaluated using Student's *t* test, with $p<0.05$ indicating statistical significance.

3. Results

3.1. Purification of CyPA and B

First, glutathione S-transferase (GST)-tagged CyPA, CyPB, the PPI inactive CyPA (CyPAΔPPI), and CyPB (CyPBΔPPI) were purified using Glutathione Sepharose 4B affinity chromatography. CyPA and CyPAΔPPI were further purified through a Superdex 200 column (Fig. S1). After the Superdex 200 gel filtration, to remove the contaminating nucleic acids, CyPB and CyPBΔPPI were further purified through MonoQ anion exchange chromatography by a continuous NaCl gradient of 50–1000 mM because CyPB has a strong affinity for nucleic acids. Each was eluted with 210–385 mM NaCl (Fig. S2). The purification scheme and purified CyPs are shown in Fig. 1. The yields of CyPA and CyPAΔPPI were approximately 3 mg from a 1-L bacterial culture. CyPA and CyPAΔPPI were >95% pure and stocked at 5 mg/mL in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. CyPB and CyPBΔPPI were stocked at 5 mg/mL in 20 mM Tris–HCl (pH 9.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT,

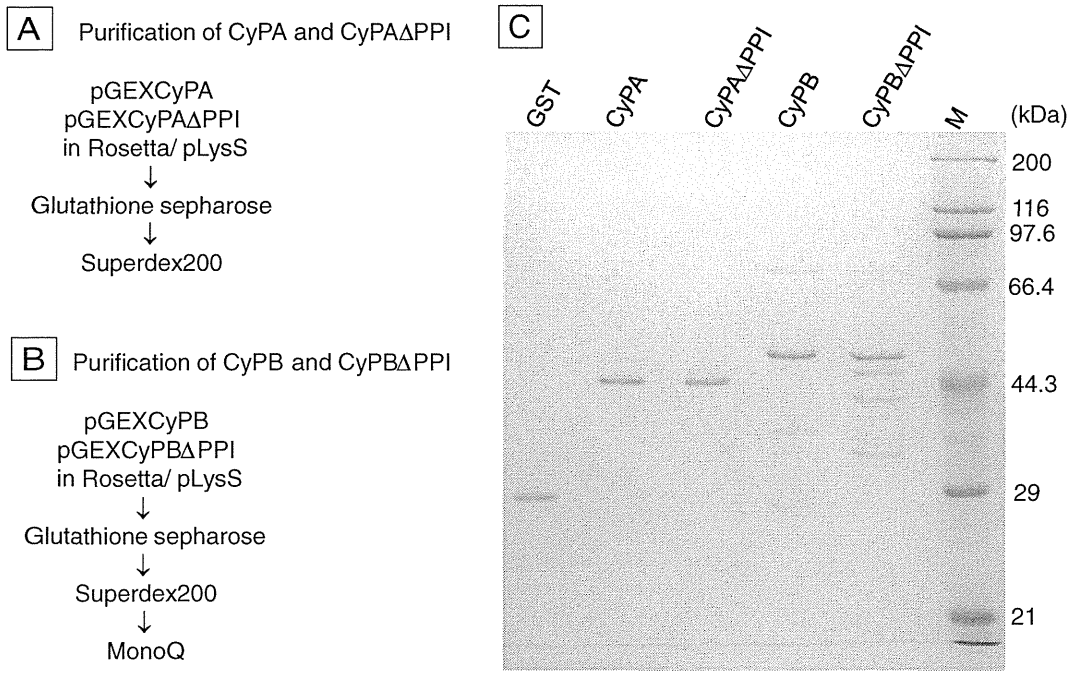

Fig. 1. Cyclophilin purification. The purification schemes of cyclophilin A (CyPA) and the peptidyl prolyl isomerase-inactive mutant protein of CyPA (CyPAΔPPI) (A), cyclophilin B (CyPB) and CyPBΔPPI (B), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (C) with 5 pmol each of purified glutathione S-transferase (GST; 28.3 kDa), GST-CyPA (44.9 kDa), GST-CyPAΔPPI (44.7 kDa), GST-CyPB (52.1 kDa), and GST-CyPBΔPPI (52 kDa) were separated through 10% SDS-PAGE and stained with Coomassie brilliant blue. The sizes of the molecular weight standards (M) are indicated on the right side of the gel. Their final elution profiles are shown in Figs. S1 and S2.

and 10% glycerol. The yields of CyPB and CyPBΔPPI were approximately 1 mg from a 1-L bacterial culture. The purities of CyPB and CyPBΔPPI were >95% and >90%, respectively.

3.2. HCV 1b and JFH1 (2a) transcription in vitro with CyPA and CyPB

The dose–response effects of CyPA and CyPB were examined using an in vitro transcription system of HCR6 (1b) and JFH1 (2a) RdRp wild type (wt). CyPA and CyPB were added to the optimal HCV in vitro transcription condition while the RNA synthesis was in the log phase [4,37]. RdRp (100 nM) was incubated with 0, 50 (ratio to RdRp: 0.5×), 100 (1×), 200 (2×), 500 (5×), and 1000 nM (10×) CyPA and CyPB, GST, or bovine serum albumin (BSA) in GTP (the initiating nucleotide) and an RNA template for 30 min, followed by elongation with ATP, CTP, and UTP for 90 min. CyPA enhancement was further tested using 2 (20×), 5 (50×), 7.5 (75×), and 10 (100×) μM because the enhancement effect of CyPA under 1 μM (10×) was unclear. Fig. S3 shows the autoradiography of HCV HCR6 (1b) and JFH1 (2a) RdRpwt with CyPA and CyPB, the graphs of which were drawn using the data from 3 independent experiments (Fig. 2).

The CyPA activation of both RdRps showed 2 reaction speeds. The first-order ratio of CyPA to HCR6 (1b) RdRpwt<50× is fitted as a linear regression curve, the equation for which is $y=0.07x$ (CyPA-to-RdRp ratio) + 0.7. The linear regression curve fitting of the ratio >50× is $y=0.4x$ (CyPA-to-RdRp ratio) − 17 when calculated from 3 points. That of CyPA to JFH1 (2a) RdRpwt is fitted to a similar linear regression, $y=0.09x$ (CyPA-to-RdRp ratio) + 0.9 (the CyPA-to-RdRp ratio<50×). HCVR6 (1b) and JFH1 (2a) RdRps were activated by 100× CyPA to 25-±0.2- and 19-±1-fold, respectively.

The CyPB activation of HCR6 (1b) RdRpwt occurred in a dose-dependent manner and fitted a sigmoid curve, and the enhancement effect reached a plateau (9.4×) at the ratio of 5×. Neither GST nor BSA enhanced HCR6 (1b) RdRpwt. CyPB, GST, and BSA did not enhance JFH1 (2a) RdRpwt (<1.5×) at the concentrations described earlier.

3.3. Effect of the PPI inactive mutant proteins of CyPA and CyPB

CyP has PPI activity. To test the contribution of PPI activity to HCV HCR6 (1b) and JFH1 (2a) RdRpwt activation, the activation effect of the PPI inactive mutant proteins, CyPAΔPPI at 100× (10 μM) and CyPBΔPPI at 2× (200 nM), were tested together with 100× (10 μM) GST and BSA (Fig. 3). CyPA enhanced JFH1 (2a) RdRpwt 17.6×, whereas CyPAΔPPI enhanced it 16.2×. This difference is statistically significant (Student's t test, $p<0.05$). CyPA enhanced HCR6 (1b) RdRpwt activity 27.7×, whereas CyPAΔPPI enhanced it 16.0×. BSA slightly inhibited both RdRps at the same concentration in this experiment. As shown in Fig. 2C and D, it can be concluded that BSA has no effect on HCV transcription. GST enhanced JFH1 (2a) RdRpwt activity 5.0×, but it did not affect HCR6 (1b) RdRpwt activity. CyPB enhanced HCR6 (1b) RdRpwt activity 2.3×, whereas CyPBΔPPI enhanced it 1.7×. This difference is also statistically significant (Student's t test, $p<0.05$). JFH1 (2a) RdRpwt was not activated by CyPB or CyPBΔPPI.

3.4. CyP activation steps of HCV transcription

The HCV transcription steps of CyP enhancement were analyzed by the sequential addition of CyPs during in vitro transcription (Fig. 4). CyPA enhanced HCR6 (1b) and JFH1 (2a) RdRpwt, whereas CyPB enhanced HCR6 (1b) RdRpwt when HCV RdRps were incubated with them from the start of transcription (initiation). The CyP effect was then tested after their addition during the elongation period after HCV RdRps was initiated with GTP. CyPA (100×; 10 μM) and CyPB (5×; 500 nM) were added to HCV RdRps after the 30-min incubation with GTP, when 3 GTPs were incorporated at the 5′ end of the products. CyPB did not enhance HCR6 (1b) or JFH1 (2a) RdRp when added during the elongation period, although it enhanced HCV RdRp when added at the start of transcription. CyPA enhanced HCR6 (1b) and JFH1 (2a) RdRp activity only 1.6× (Student's t test, $p<0.05$) and 2.1× ($p<0.01$), respectively, when added during the elongation step. These results suggest that CyPA and CyPB activated only the transcription initiation step of HCV RdRps.

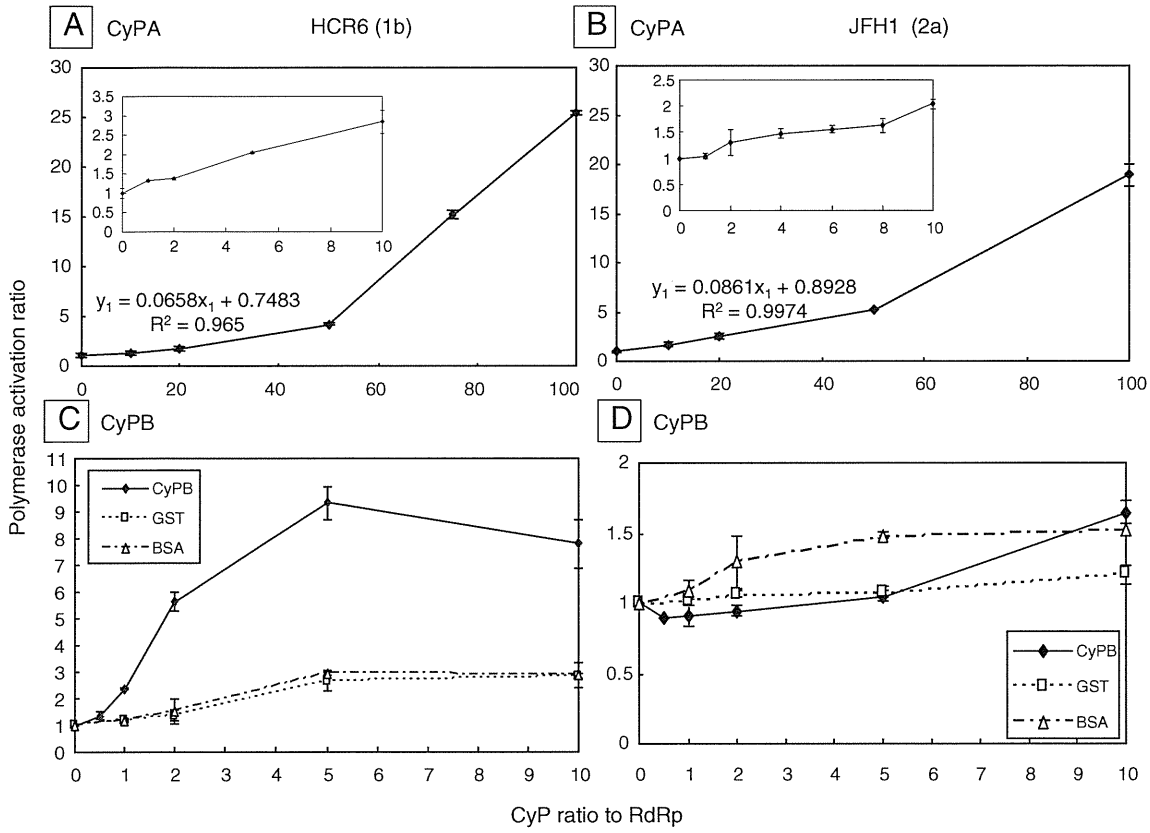

Fig. 2. Dose–response curve of cyclophilin A (CyPA) and cyclophilin B (CyPB) in hepatitis C virus (HCV) transcription in vitro. The dose–response curve of the HCV RdRp activation of CyPA in HCR6 (1b) RdRpwt (A) and JFH1 (2a) RdRpwt (B) CyPB in HCR6 (1b) RdRpwt (C) and JFH1 (2a) RdRpwt was drawn from the image analysis of Fig. S3. Insets A and B indicate that of 0, 0.5×, 1×, 2×, 5×, and 10× of CyPA to RdRp. The first-order ratio of the curves of A and B were fit by linear regression, and the calculated equations are indicated in the graph. The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements.

The effects of 75× CyPA and 5× CyPB on the RNA-binding activity of HCR6 (1b) and JFH1 (2a) RdRp were then tested (Fig. 4E). The effects of HCR6 (1b) and JFH1 (2a) RdRp with CyPA were 10.1-±0.56- and 6.6-±0.68-fold of that without CyPA, respectively. The effect of HCR6 (1b) RdRp with CyPB was 3.1-±0.3-fold of that without CyPB. The RNA-binding activity of HCV RdRps was thus enhanced by the addition of CyPA and CyPB.

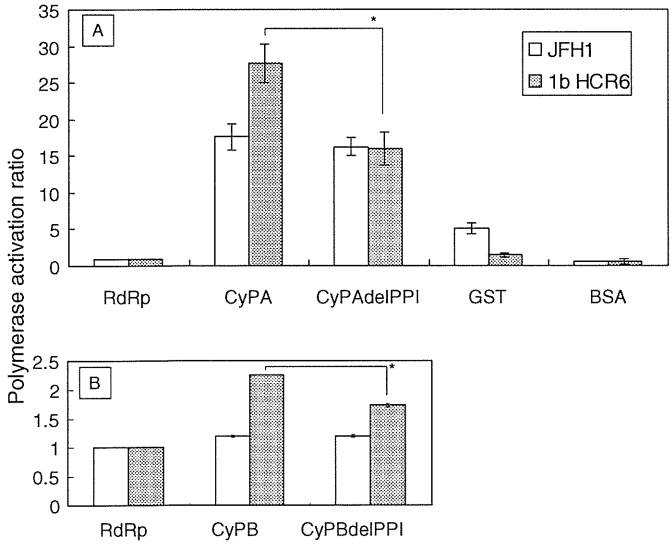

Fig. 3. Effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) with and without peptidyl prolyl isomerases activity on hepatitis C virus (HCV) JFH1 (2a) and HCR6 (1b) RdRp. HCV HCR6 (1b) and JFH1 (2a) RdRpwt (100 nM) were incubated with 100× (10 μM) of CyPA, CyPAΔPPI, glutathione S-transferase (GST), and bovine serum albumin (BSA) (A). HCV RdRps were incubated with 5× (500 nM) of CyPB, CyPBΔPPI, GST, and BSA (B). The mean relative polymerase activity and standard deviation (error bar) were calculated from 3 independent measurements. *$p<0.01$ (Student's t test).

3.5. Effect of CyP activation on RdRp of various HCV genotypes

The CsA sensitivity differed among the HCV genotypes [41]. Therefore, we tested the effects of CyPA and CyPB activation on NN (1b), H77 (1a), RMT (1a), and J6CF (2a) RdRp (Fig. 5). RdRp activity was compared with and without 50× (5 μM) CyPA and 5× (500 nM) CyPB. At their respective concentrations, CyPA activated all of the tested HCV RdRps by 3.9–5.3×, but CyPB activated only 1b RdRps (8–10×). CyPB slightly activated J6CF (2a) RdRp (approximately 4×), but it did not activate the 1a or JFH1 (2a) RdRps (1.4–1.8×).

4. Discussion

Since CsA was discovered to inhibit HCV infection [23–26], the CyP pathway contributing to HCV replication has been proposed as a potential stratagem for controlling HCV infection. Reports about the roles of CyPA in HCV replication via NS5A have been accumulating [33–35,42–44]. However, the effect of CyP inhibitors varied on the RNA-binding activity of NS5B [41,45], and DEBIO-025 decreased CyPB levels in patients [46]. Controversial results of CyPA and CyPB knockout experiments on HCV replicon activity were reported [29,30,47]. Therefore, the effects of CyPA and CyPB on HCV RdRp were carefully analyzed again in vitro.

In this study, we demonstrated that CyPA and CyPB activated HCV 1b RdRp in vitro by completely different kinetics using purified CyPs

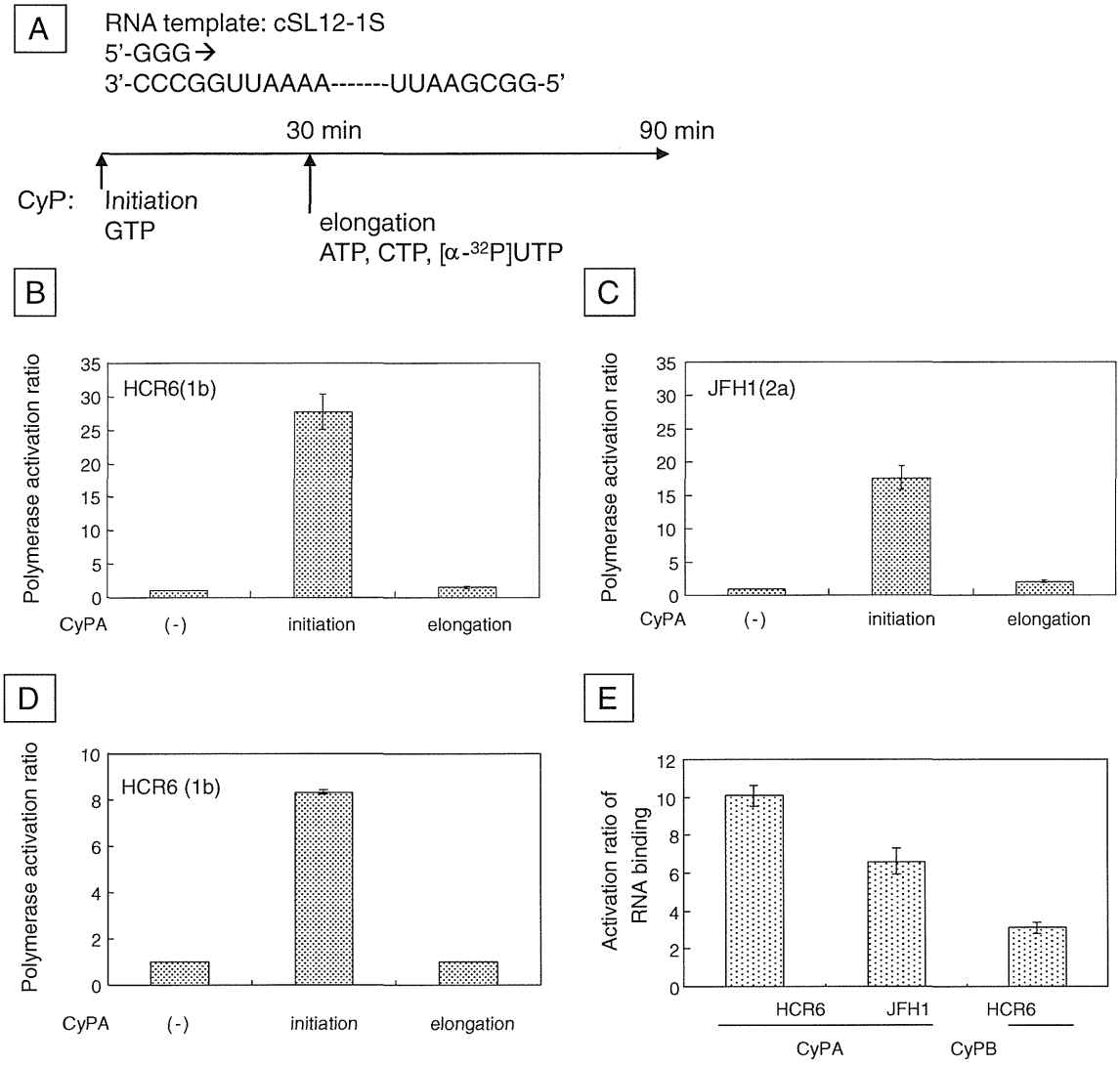

Fig. 4. Hepatitis C virus (HCV) RdRp activation effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) on transcription initiation and elongation. The polymerase activation effect of the timing of the CyPA or CyPB addition was examined. The sequence of the model RNA template (SL12-1S) and experimental design are shown in A. CyPA 100× (10 μM) was incubated with HCR6 (1b) RdRpwt (A) and JFH1 (2a) (B) RdRp during preincubation with 0.5 mM GTP (initiation) or after preincubation (elongation). CyPB 5× (500 nM) was incubated with HCVR6 (1b) RdRpwt during preincubation with 0.5 mM GTP (initiation) or after the preincubation (elongation) (C). The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements. The effect of the 100× CyPA and 5× CyPB on RNA template binding was examined (E).

and HCV RdRps (Fig. 2), which indicated that the mechanism of their HCV RdRp activation differed despite their similar structures [48–50]. Kinetic analysis of CyPA on HCR6 (1b) and JFH1 (2a) RdRp indicated that it had a similar activation mechanism on both HCV RdRps. CyPA did not activate HCV RdRp at low concentrations, but it did activate it at >50× molar excess to it. The unusual dose of CyPA activating HCV RdRp (Fig. 2) postulates that HCV RdRp may be surrounded by CyPA in vitro and factors involving CyPA and HCV RdRp interaction, such as NS5A, in the HCV replication complex of the infected cells [27,28,31,36,51–53] because the interaction of CyPA and HCV RdRp was weak (Fig. S4).

Although some controversial results were obtained from those of Heck et al. [54], the studies agree that CyPB also activated HCV 1b RdRp in vitro. The activation kinetics of CyPB on HCR6 (1b) RdRp showed a sigmoid-like curve (Fig. 2) that suggested an allosteric effect of CyPB on RdRp activity. CyPB may interact with HCV RdRp as a cofactor and directly activate HCR6 (1b) RdRp. The HCV RdRp–CyPB complex was likely to interact more with CyPB, and its activation plateaued at the CyPB/RdRp ratio of 5:1 (Fig. 2C). The CyPB activation curves of Heck et al. [54] also plateaued. These data from the 2 independent groups support the weak interaction between CyPB and HCV 1b RdRp (Fig. S4).

CyPA did not show genotype specificity in the current study (Fig. 5A), a finding that agrees with those of CyPA knockdown, DEBIO-025, and CsA experiments [30,43,55]. CyPB activation showed genotype specificity (Fig. 5B) [54]; CyPB activated 1b and J6CF (2a) RdRp but did not activate 1a or JFH1 (2a) RdRp. Both reports agreed with the finding that JFH1 (2a) subgenomic replicon was independent of CyPB [41]. Although mutations accumulated in the NS5A region of CsA- or DEBIO-025-resistant HCV replicons, some mutations were found in the NS5B region [18,27,28,33,45].

Another controversial result between that of Heck et al. [54] and ours is the Mg^{2+}-dependency of the CyPB activation. The Mg^{2+} concentration in cells is 14–20 mM, and Mg^{2+} ions are distributed almost equally throughout the nuclei, mitochondria, and cytosol/endoplasmic reticulum [56]. The Mn^{2+} concentration in cells varies from report to report [57,58]. The optimal Mn^{2+} and Mg^{2+} concentrations in the HCV in vitro transcription used in this study were

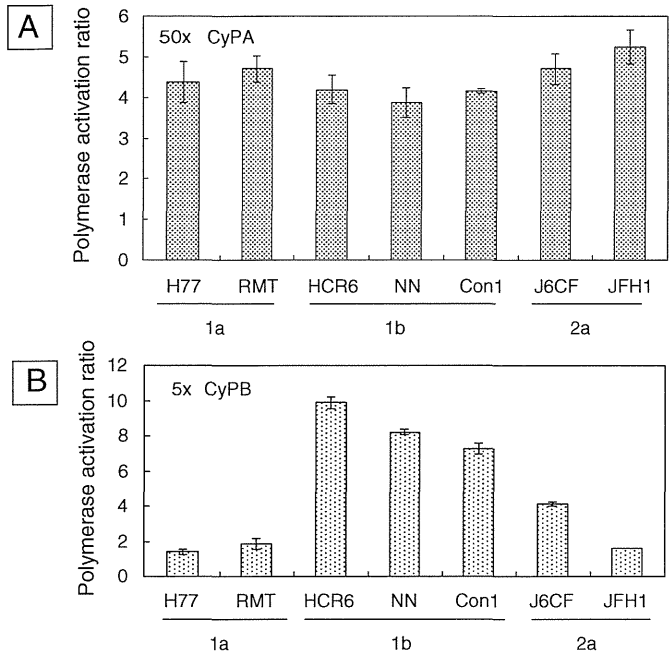

Fig. 5. Activation effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) on hepatitis C virus (HCV) RNA polymerase of genotypes 1a, 1b, and 2a. The polymerase activation effects of CyPA and CyPB on HCV 1a (H77 and RMT), 1b (HCR6, NN, and Con1), and 2a (J6CF and JFH1) were examined. HCV RdRp (100 nM) was incubated with 50× CyPA and 5× CyPB. The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements.

different from the physiological concentrations in cells [4,37]. However, under the optimal HCV transcription condition, HCV RdRp activation was observed by CyPA and CyPB (Fig. 1).

The amount of CyPA varies by cell type [59]. In some cells, CyPB may also contribute to HCV 1b replication because it localizes in the endoplasmic reticulum and plasma membranes [60,61], which form a membrane web in which an HCV replication complex exists [1].

PPI activity of CyPs is essential for HCV replicon activation [32,53]. CyP inhibitors (DEBIO-025, NIM811, and SCY-635) inhibit PPI activity. The PPI activity of CyPA contributed to HCV RdRp activation and CyP-NS5A binding [36]. The PPI activity of CyPA partly contributed to the activation of HCR6 (1b) RdRpwt in vitro (Fig. 3A, $p<0.01$). The PPI activity of CyPB may not be essential for RdRp activation because the activation ratio was not large between CyPB and CyPBΔPPI, although the experiment showed a statistically significant difference (Fig. 3B). There may be differences in the RdRp activation mechanisms of CyPA with and without PPI activity. This finding will help with the development of new CyPA inhibitors that target domains other than PPI.

The mechanism of HCV RdRp activation by CyPs is not clear. In the least, CyPA and CyPB enhanced the early stage of HCV transcription, including the template RNA binding of HCV RdRp (Fig. 4) [29,41,45]. The productive template-polymerase binding is the late-limiting step of transcription initiation by HCV RdRp in vitro, and a small fraction of HCV RdRp was active in vitro [62,63]. CyP may enhance this step on many HCV RdRp molecules to show apparent activation of RdRp in vitro.

Considering the controversial reports on CyP and HCV replication [29,33,35,41,43,44], it can be concluded that CyPA is the major factor of HCV genome replication and that the activation of HCV RdRp may require other factors such as NS5A to condense CyPA around the HCV RdRp. Although many HCV treatment approaches have been applied in addition to Peg-IFN, ribavirin, and NS3/NS4a protease inhibitor [64–67], more effort has to be made to ensure an HCV cure. This study and that of Heck et al. [54] demonstrated similar activation kinetics and genotype specificity of CyPB activation (Figs. 2 and 5). CyPB also has the potential to activate HCV 1b genome replication in a limited condition, and it should also be included as the target of inhibitor development because HCV 1b is the genotype that is most resistant to treatment [13].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2012.08.017.

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Japanese Reference Panel of Blood Specimens for Evaluation of Hepatitis C Virus RNA and Core Antigen Quantitative Assays

Asako Murayama,[a] Nao Sugiyama,[a] Koichi Watashi,[a] Takahiro Masaki,[a] Ryosuke Suzuki,[a] Hideki Aizaki,[a] Toshiaki Mizuochi,[b] Takaji Wakita,[a] and Takanobu Kato[a]

Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan,[a] and Department of Research on Blood and Biological Products, National Institute of Infectious Diseases, Tokyo, Japan[b]

An accurate and reliable quantitative assay for hepatitis C virus (HCV) is essential for measuring viral propagation and the efficacy of antiviral therapy. There is a growing need for domestic reference panels for evaluation of clinical assay kits because the performance of these kits may vary with region-specific genotypes or polymorphisms. In this study, we established a reference panel by selecting 80 donated blood specimens in Japan that tested positive for HCV. Using this panel, we quantified HCV viral loads using two HCV RNA kits and five core antigen (Ag) kits currently available in Japan. The data from the two HCV RNA assay kits showed excellent correlation. All RNA titers were distributed evenly across a range from 3 to 7 log IU/ml. Although the data from the five core Ag kits also correlated with RNA titers, the sensitivities of individual kits were not sufficient to quantify viral load in all samples. As calculated by the correlation with RNA titers, the theoretical lower limits of detection by these core Ag assays were higher than those for the detection of RNA. Moreover, in several samples in our panel, core Ag levels were underestimated compared to RNA titers. Sequence analysis in the HCV core region suggested that polymorphisms at amino acids 47 to 49 of the core Ag were responsible for this underestimation. The panel established in this study will be useful for estimating the quality of currently available and upcoming HCV assay kits; such quality control is essential for clinical usage of these kits.

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide (15). There is no protective vaccine against this virus, and once an individual is infected, HCV often establishes persistent infection and leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (9). The most widely used therapy for HCV infection is the combined administration of pegylated alpha interferon and ribavirin (29). However, this treatment is problematic, as it has limited efficacy, high cost, and severe adverse effects (8, 25). To estimate the outcome of antiviral therapy, and to understand the state of viral propagation, it is important to determine the HCV viral load in chronic hepatitis C patients by the use of accurate and reliable HCV quantitative assays (9, 14). For this purpose, several commercial assay kits for HCV RNA and core antigen (Ag) quantification are currently used in Japan. For quantification of HCV RNA levels, two real-time quantitative reverse transcription-PCR (qRT-PCR)-based assay kits are available, including the COBAS AmpliPrep/COBAS TaqMan HCV test (CAP/CTM-RNA; Roche Diagnostics, Tokyo, Japan) and the Abbott RealTime HCV test (ART-RNA; Abbott Japan, Tokyo, Japan). These assays are known to have high sensitivity and a wide dynamic range, but they require technical skill and attention to maintaining the specified conditions (4–6, 16, 24, 33–35). Alternatively, HCV viremia can be quantified by assessment of HCV core Ag level (1–3, 7, 10, 12, 13, 17–22, 27, 30–32). Five HCV core Ag assay kits are commercially available in Japan, including Architect HCV Ag (Architect-Ag; Abbott Japan), Lumipulse Ortho HCV Ag (Lumipulse-Ag; Fujirebio, Tokyo, Japan), Lumispot Eiken HCV Ag (Lumispot-Ag; Eiken Chemical, Tokyo, Japan), the Ortho HCV Ag ELISA test (ELISA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan), and the Ortho HCV Ag IRMA test (IRMA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan). These assays have some disadvantages compared to those measuring HCV RNA (notably, low sensitivity and narrow range of quantification) but also have some advantages (including ease of use, reduced risk of contamination, reduced cost, and reliability even with samples stored at room temperature for extended periods of time [1, 32]). Although core Ag levels are thought to be related closely to HCV RNA titers, the correlation and linearity of core Ag levels have not yet been fully evaluated. In addition, these quantitative parameters are known to be affected by nucleotide and amino acid sequences at the target regions of the assays (5, 6, 28, 34), and this sequence variation depends on genotypes or predominant strains in specific geographical regions.

In this study, we established a Japanese reference panel of samples for evaluation of HCV RNA and core Ag levels by collecting donated blood specimens that tested positive for HCV RNA and anti-HCV antibodies. Using this reference panel, we evaluated the HCV loads in these specimens with two HCV RNA assay kits and five core Ag assay kits and assessed correlations among the data generated by these kits.

MATERIALS AND METHODS

Preparation of reference panel. To establish a reference panel for HCV quantitative assays, a total of 80 donated plasma samples were selected. All of these specimens, supplied by the Japanese Red Cross Blood Centers, tested positive for the presence of HCV RNA and anti-HCV antibodies. These samples, collected in Japan from May to September of 2007, were obtained from Japanese blood donor volunteers in various regions of

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Address correspondence to Takanobu Kato, takato@nih.go.jp.
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