201125043A

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２３年度　総括研究報告書

研究代表者　　渡士　幸一

平成２４（２０１２）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を標的とした新規治療法の開発を目的とし、（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）さらに抗HCV効果の高いmiRNA阻害剤の探索、（3）in vivo HCV感染系におけるmiRNA阻害剤の効果の検証、を行った。その結果、miRNAによるHCV複製亢進作用にはAGO2が重要であること、miRNA阻害剤によるHCV複製抑制とmiRNA-AGO2解離が相関していることを見出した。TPFは少なくとも今回のin vivo HCV感染モデルにおいて顕著な抗HCV効果は示さなかったが、これはTPFがマウス体内において速やかに代謝／排泄されるためであることを示唆する結果を得た。これらの情報をもとに、今後抗HCV効果が高いことだけではなく、体内動態が良好であるという指標を用いて、TPF誘導体あるいはmiRNA阻害剤の取得を目指す。

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。本研究では宿主マイクロRNA（miRNA)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。しかしmiRNA経路を利用した創薬応用は、いまだどの疾患に対してもおこなわれていない。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はじめており、細胞において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。本研究では主にこの化合物をプラットフォームとして、miRNAによるHCV複製機構の解析をおこなうとともに、抗HCV剤開発をおこなう。具体的には、（1）miRNA阻害剤TPFによるHCV複製抑制メカニズムの解析、（2）さらに抗HCV作用の高い、毒性の少ない化合物の探索、（3）TPFのin vivoにおける抗HCV効果、毒性の評価、を行った。

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>

HeLa細胞に合成miR-122をトランスフェクションした後、in vitro合成したHCV遺伝子型1b型RNAをエレクトロポレーションで導入し、HCVゲノム複製が持続的に行われている細胞をG418 1000μg/mlで選択した。3週間後に生き残った細胞をクリスタルバイオレットで染色することにより、HCVゲノム複製活性を計測した。またAGO2に対するsiRNAをトランスフェクションした細胞あるいはTPF処理細胞を用いることにより、AGO2の効果を検証した。またTPFを処理した、HCVレプリコンを有するHuh-7細胞中のHCV RNAおよびタンパク質をreal time RT-PCR法およびイムノブロット法を用いて解析した。

また細胞溶解液にビオチン化miR-122を加え、ストレプトアビジン化ビーズ沈降物中のAGO2をイムノブロット法で検出することにより、miR-122とAGO2の結合を評価した。

(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より購入した8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ゲノム複製は、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) in vivo HCV感染系におけるmiRNA阻害剤の効果の検証

ヒト肝細胞を移植したuPA TG/SCIDマウスへのHCV感染は（株）フェニックスバイオの標準プロトコールに則った。HCV感染後、血中HCV RNA量が$1x10^6$コピー/ml以上で安定した後にACF 0.5 mg/kgを筋肉注射で1日1回、2週間投与した。さらに2週間ごとにACF投与量を段階的に上昇させ、8 mg/kgの2週間投与までおこなった。マウスへの毒性は、体重測定および血中ヒトアルブミン定量により評価した。また血中HCV RNAをreal time RT-PCR法で定量することにより抗ウイルス効果の有無を調べた。

また、1) ACFとddYマウス血清をin vitroで混合し、37°Cで30, 60, 120, 180分間インキュベーション後のACF濃度、2) ACF 20 μg/bodyをddYマウスに投与し、5, 15, 30, 60, 120分後の血中および肝臓内ACF濃度を、蛍光-HPLC分析法により測定した。

（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. miR-122によるHCV複製亢進機能における argonaute 2 (AGO2)の役割の解析

内在性miR-122発現がほとんどないHeLa細胞ではHCV遺伝子型1株のHCVゲノム複製がほとんど見られないが、これにmiR-122を過剰発現することによりゲノム複製活性が著明に上昇すること、さらにこれにAGO2に対するsiRNAあるいはTPFを持続的に処理することにより上昇したHCV複製が大きく低下することが認められた。またHCVレプリコン細胞にTPFを処理することにより、HCVゲノムRNAが有意に低下することが観察された。

2. TPFの抗HCV活性におけるmiR-122とAGO2の解離

HCVレプリコン細胞にTPFを処理し抗HCV効果を発揮している時に、miR-122とAGO2の解離がどのように変化しているかを調べた。その結果、TPF処理によりmiR-122とAGO2の結合が減弱していること、また抗HCV効果をもたないTPF誘導体ではこの解離が見られないことが示された。

(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、8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。

(3) in vivo HCV感染系におけるmiRNA阻害剤の効果の検証

ヒト肝細胞を移植したuPA TG/SCIDマウスにHCVを感染させ、その後にacriflavine (ACF: TPFとproflavineの合剤)を投与することにより、in vivoでのACFの抗HCV効果の有無を検証した。ACF濃度は0.5 mg/kgから2週間ごとに段階的に1 mg/kg, 2 mg/kg, 4 mg/kg, 8 mg/kgと増加させていき、10週間後まで経時的に血中HCV量を定量した。しかしながら少なくとも本実験においては血中HCV量は大きな変化を認めなかった。

また抗HCV効果が見られなかった原因を推定するために、1) ACFとddYマウス血清をin vitroで混合しACF濃度を測ったが、経時的なACF濃度濃度の減少は認めなかった。しかし2) ACFをddYマウスに投与し、経時的な血中および肝臓内ACF濃度を測定した

ところ、ACF濃度は投与5分後で検出限界程度にまで顕著に低下した。

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

抗AGO2に対するsiRNAあるいはTPFを用いた結果より、miR-122によるHCV複製亢進にはAGO2とmiR-122の会合が重要であることが示唆された。またTPFによる抗HCV効果は、miR-122-AGO2結合の解離を伴うことが考えられた。以上の結果は、AGO2を含むRNA-induced silencing complex (RISC)複合体が、miRNA本来の遺伝子サイレンシング機能以外にも役割を果たすことを示唆するものである。

(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効果が高かったため、今後さらなる誘導体解析が必要である。

(3) in vivo HCV感染系におけるmiRNA阻害剤の効果の検証

ヒト肝細胞を移植したuPA TG/SCIDマウスにおいてACFは顕著な抗HCV効果を示さなかったが、その後のddYマウスを用いた解析により、1) ACFは血中で物理的には安定である、2) しかしながらACFはマウスに投与後、体内で急速に濃度が低下する、ことが示唆された。マウス体内での急速な濃度低下により、ACFの代謝／排泄は非常に速いことが示唆された。今後、代謝／排泄を遅らせたACF誘導体を開発する必要があると考えられる。

E. 結論

miRNA阻害剤TPFを用いて、miRNAによるHCV複製亢進作用にはAGO2が重要であること、miRNA阻害剤によるHCV複製抑制とmiRNA-AGO2解離が相関していることを見出した。またTPFを含む化合物はin vivo HCV感染系において抗HCV作用を示さなかったが、これはこの化合物がマウス体内において速やかに代謝／排泄されるためであると考えられた。

今後化合物の特性をさらに至適化することによりin vivoで抗HCV効果をもつ化合物の取得を目指す。

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. 知的所有権の出願・取得状況

1. 特許取得

なし。

2. 実用新案登録

なし。

3. その他

なし。

II. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Morohashi K*, Sahara H, Watashi K*, Iwabata K, Sunoki T, Kuramochi K, Takakusagi K, Miyashita H, Sato N, Tanabe A, Shimotohno K, Kobayashi S, Sakaguchi K, Sugawara F (* equally contributed)	Cyclosporin A associated helicase-like protein facilitates the association of hepatitis C virus RNA polymerase with its cellular cyclophilin B	PLoS One	6	e18285	2011
Mohammed TAS, Aoyama H, Sugita K, Watashi K, Wakita T, Hamasaki T, Okamoto M, Urata Y, Hashimoto Y, Baba M	Potent and selective inhibition of hepatitis C virus replication by novel phenanthridinone derivatives	Biochem Biophys Res Commun	415	714-719	2011

III. 研究成果の刊行物・別刷

Cyclosporin A Associated Helicase-Like Protein Facilitates the Association of Hepatitis C Virus RNA Polymerase with Its Cellular Cyclophilin B

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Abstract

Background: Cyclosporin A (CsA) is well known as an immunosuppressive drug useful for allogeneic transplantation. It has been reported that CsA inhibits hepatitis C virus (HCV) genome replication, which indicates that cellular targets of CsA regulate the viral replication. However, the regulation mechanisms of HCV replication governed by CsA target proteins have not been fully understood.

Principal Findings: Here we show a chemical biology approach that elucidates a novel mechanism of HCV replication. We developed a phage display screening to investigate compound-peptide interaction and identified a novel cellular target molecule of CsA. This protein, named CsA associated helicase-like protein (CAHL), possessed RNA-dependent ATPase activity that was negated by treatment with CsA. The downregulation of CAHL in the cells resulted in a decrease of HCV genome replication. CAHL formed a complex with HCV-derived RNA polymerase NS5B and host-derived cyclophilin B (CyPB), known as a cellular cofactor for HCV replication, to regulate NS5B-CyPB interaction.

Conclusions: We found a cellular factor, CAHL, as CsA associated helicase-like protein, which would form trimer complex with CyPB and NS5B of HCV. The strategy using a chemical compound and identifying its target molecule by our phage display analysis is useful to reveal a novel mechanism underlying cellular and viral physiology.

Citation: Morohashi K, Sahara H, Watashi K, Iwabata K, Sunoki T, et al. (2011) Cyclosporin A Associated Helicase-Like Protein Facilitates the Association of Hepatitis C Virus RNA Polymerase with Its Cellular Cyclophilin B. PLoS ONE 6(4): e18285. doi:10.1371/journal.pone.0018285

Editor: Robyn Klein, Washington University, United States of America

Received April 15, 2010; **Accepted** March 2, 2011; **Published** April 29, 2011

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Funding: This work was supported by The Promotion and Mutual Aid Corporation for Private Schools of Japan, Grant-in-Aid for Matching Fund Subsidy for Private Universities. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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☯ These authors contributed equally to this work.

Introduction

Cyclosporin A (CsA) possesses immunosuppressive effects and is widely used for allogeneic transplantation [1]. These therapeutic effects of CsA, in particular downregulation of interleukin 2 (IL-2) production by T cells, are considered to be responsible for the suppression of immunological events via cellular immunology [2,3]. Its mechanism is widely believed to include CsA binding to its primary cytoplasmic receptor cyclophilin A (CyPA). This CsA/CyPA complex inhibits the phosphatase activity of calcineurin, which is essential for the activation of nuclear factor of activated T cells (NFAT) transcription factors and their downstream cytokine production [2–5]. The cyclophilins (CyP), identified as cytoplasmic receptors for CsA are a family of peptidylprolyl cis-trans isomerases (PPIase) and include more than ten subtypes [6–8]. Recently, it was reported that several CyPs regulated hepatitis C virus (HCV) replication; CyPA binds to HCV NS5A and NS5B proteins. CyPB also interacted with HCV NS5A and NS5B [9–11]. The interaction of CyPB stimulates the RNA binding activity of NS5B. These viral-cellular interaction mechanisms were revealed by a chemical biological analysis focusing on an anti-viral characteristic of CsA. However, it has not been fully understood how a series of CsA-target proteins regulate HCV replication. We obtained the data suggesting the possibility that CsA target factor(s) other than CyP family also modify HCV replication.

To exploit a novel drug target is a challenging but a powerful strategy to elucidate unknown aspects of cellular physiology that are modified by the compound. In this study, we identify a CsA binding factor by a phage display method. There are various methods to isolate targets of small molecules. Most of the methods, however, require tagged small molecules for screening to separate the drug and protein complex. The steps to synthesize tagged small molecules are technically limited in the case of complicated molecules such as CsA. To overcome this limitation, we recently developed a labeling method that can be theoretically utilized for

any chemical substance [12]. A highly reactive carbene induced by UV irradiation reacts with CsA, resulting in the production of immobilized CsA in a nonspecific manner. By using the photoaffinity method, we successfully immobilized CsA on resins and performed phage display screening. This method cloned a CsA associated helicase-like protein, which we termed CAHL, and this protein was shown to interact with HCV replication machinery. Our result presents an example for the chemical biological method that could facilitate to reveal a mechanism of viral-cellular interaction.

Results

Phage display screening with immobilized CsA isolated CsA associated helicase-like protein, CAHL

To explore CsA binding proteins, we applied a chemical biology approach. In general, small molecule is necessary to be chemically modified such as biotinylated to be immobilized on solid surface for isolation of binding proteins. However, due to the structural complexity of CsA, it is technically challenging to chemically modify a certain residue of CsA. Therefore, we took advantage of photoaffinity coupling method, which we previously developed [12]. The highly reactive carbene induced by UV irradiation reacted with CsA, resulting in the production of immobilized CsA on solid surface in a nonspecific manner (Fig. 1A). We performed phage display screening with multiple cycles that consist of binding, washing, recovery and amplification (Fig. 1B). We used phage particles randomly displayed 12 amino acids as a library [13]. Through the screening cycles, the ratios of eluted phage particles associated with CsA-immobilized resins comparing to input were dramatically increased (Fig. 1C). We randomly picked up 22 single phage clones from the sixth panning elution (Table S1). Five out of the 22 phage clones were identical, and we called it phage #13. In order to validate the binding specificity of the phage, we amplified phage #13 and measure the ratio of eluted phage titer with CsA and mock resins, which were treated with MeOH to block photoaffinity reaction. The ratio of the phage #13 was 3.75, whereas randomly picked up phage was 1.00. These results indicated that the phage #13 specifically associated with CsA-immobilized resins.

CAHL has an RNA-dependent ATP hydrolysis activity

Phage #13 was predicted to display amino acids, LVFGTLLG-QLRA, in the carboxyl terminus of its phage-coat protein, which is responsible for interaction with CsA. We searched the protein database to find proteins that showed similarities to the LVFGTLLGQLRA sequence. As a result of the search, we found a protein with a sequence identical to LLGQLRA, encoded by a gene accession number NM_022828 in the NCBI database. NM_022828 is predicted to encodes 1430 amino acid protein that has a couple of conserved domains, such as DEXHc helicase, RNA-dependent ATPase and ankyrin repeat (Fig. 2A; Fig S1). LLGQLRA sequence is located in the middle region of the protein (amino acids 940–946), where is no known conserved motif is found (Fig. 2A). Since it has not been reported on its biological functions, hereafter we refer to the NM_022828 as CsA-associated helicase-like protein, CAHL. To confirm the interaction between CAHL and CsA, we prepared a recombinant C-terminal protein of CAHL (named CAHL-C) that consisted of amino acids 761 to 1430 (Fig. 2B) including LLGQLRA motif, and performed surface plasmon resonance (SPR) analysis. It was difficult to use a full-length CAHL for *in vitro* pull-down assays since obtaining enough amount of full-length CAHL for SPR was technically challenging due to high insolubility. Considering that CsA binding sequence

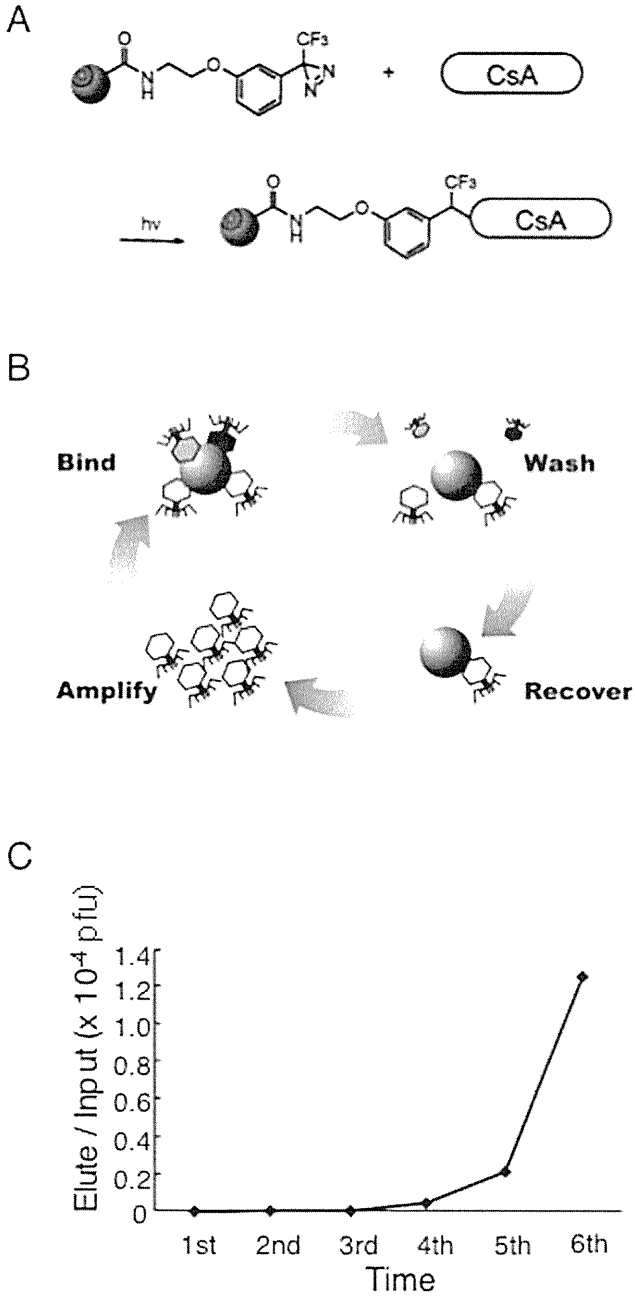

Figure 1. Immobilization of CsA and phage display screening. (A) A schematic diagram of CsA immobilization on photoaffinity resins. (B) Procedure of phage display screening. (C) Relative enrichment of phage particles. Relative enrichment was determined by the relationship between phage titer of elution from a CsA immobilized resins and input.
doi:10.1371/journal.pone.0018285.g001

found by the phage display screening is located C-terminus of CAHL, we used CAHL-C protein. A specific binding response with CsA was observed (KD = 1.2×10^{-7} M), whereas those with FK506, which is an immunosuppressant and has no HCV-inhibitory activity, were significantly weak (KD = 2.5×10^{-6} M) (Fig. 2C). Since CAHL was predicted to be RNA-dependent ATPase based on conserved domains (Fig. 2A), we measured the ATPase activity of CAHL in the presence and absence of RNA. As shown in Fig.2D, RNA-dependent ATP hydrolytic activity of CAHL-C was clearly observed, and this activity was suppressed in

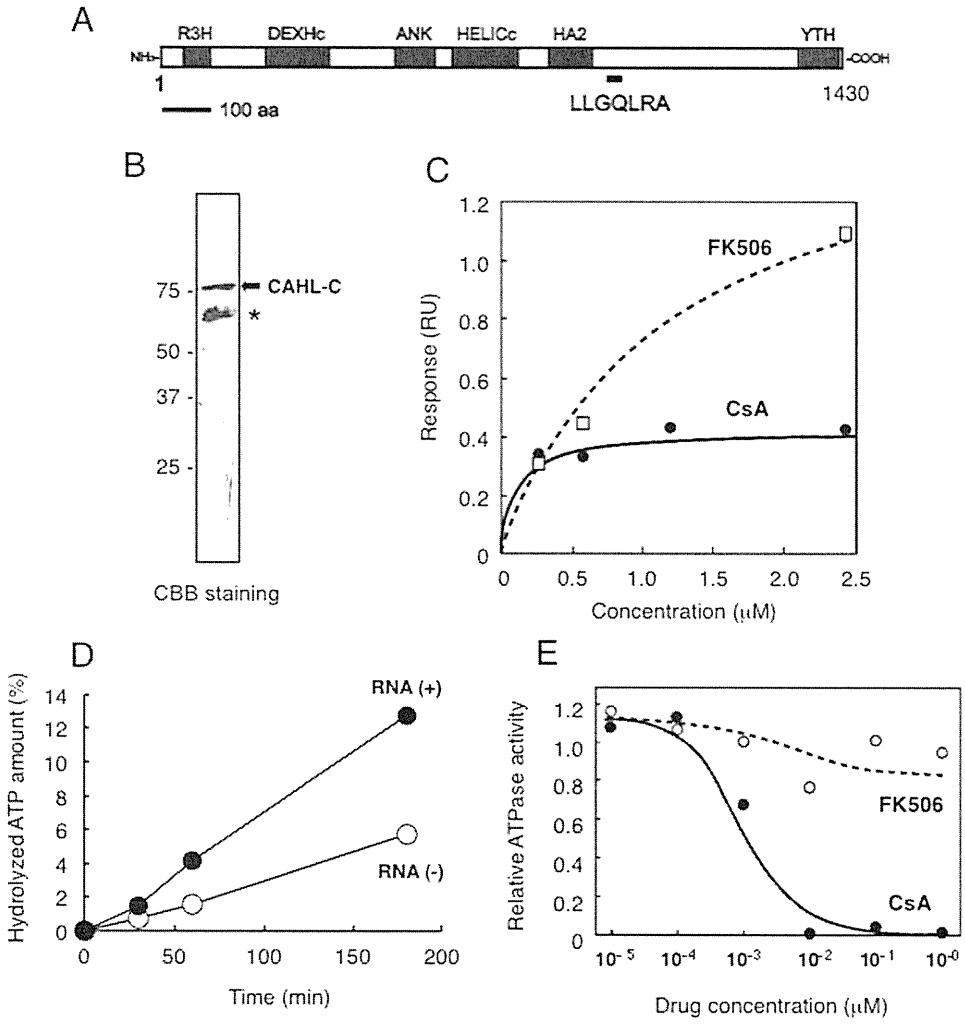

Figure 2. Cloning of CsA associated helicase-like protein (CAHL) by phage display screening. (A) Schematic representation of CAHL protein. R3H (*cd*02325), DEXHc (*cd*00269), ANK (*cd*00204), HELICc (*smart*00490), HA2 (*pfam*04408) and YTH (*pfam*04146) motifs were found by a CD search (http://www.ncbi.nlm.nih.gov/Structure/edd/wrpsb.cgi). Underline indicates a region of the LLGQRA amino acid sequence identical to the CsA-associated sequence displayed on phage #13. (B) Purified recombinant CAHL-C protein was confirmed by SDS-PAGE analysis (arrow). Asterisk indicates degraded products. (C) A kinetic plot and binding isotherm for binding of CsA (closed circle) and FK506 (opened square) to CAHL-C sensor chips in concentrations ranging from 0.25 to 2.5 mM. The estimated KD value of interaction between CAHL-C and CsA or FK506 was 1.2×10^{-7} or 2.5×10^{-6} M, respectively. (D) RNA-dependent ATP hydrolytic activities of CAHL. Filled and open circles indicate ATP hydrolytic activities of CAHL in the presence (closed circle) or absence (opened circle) of total RNA extracted from liver cells, respectively. (E) CsA inhibitory effects on ATP hydrolytic activities of CAHL. Filled and open circles indicate ATP hydrolytic activities with CsA (closed circle) or FK506 (opened circle), respectively.
doi:10.1371/journal.pone.0018285.g002

a dose-dependent manner when CsA, but not FK506 (Fig. 2E). A difference of inhibitory effects of CsA and FK506 on CAHL-C hydrolytic activity is more than 2-orders of magnitude. Considering that the difference of *KD*s of CsA and FK506 values measured by SPR, CsA association with CAHL would significantly affect on the activity. These results indicate that CAHL had RNA-dependent ATPase activity and was specifically inhibited by CsA.

CAHL is localized in ER and its expression is up-regulated by TNF-α treatment

Since biological functions of CAHL were unknown, we investigated the biological background of the CAHL gene. We first performed RNA blotting analysis and RT-PCR using normal human tissues and tumor cells. As shown in Fig. 3A, CAHL-transcripts with approximately 1.6 kbp were detected in both human hepatoma Huh-7 cells and MH-14 cells, which do not and do carry the HCV subgenome replicon, respectively, whereas much less was detected in normal liver tissues. RT-PCR analysis revealed that in other normal tissues (though not testis) little or no expression of CAHL was observed as compared the house-keeping gene G6PDH, whereas clear expression of it was detected in all the tumor cell lines examined (Fig. 3B). Since the CAHL expression was very little in the normal liver tissues, a question was arisen: how is CAHL expression regulated? It is possible that CAHL could be induced by inflammation caused by virus infections. To test the hypothesis, we observed whether CAHL expressions are induced by inflammatory signals. Indeed, CAHL in normal liver cells was upregulated in the presence of a proinflammatory cytokine, tumor necrosis factor (TNF)-α with dose dependent manner (Fig. 3C), suggesting that CAHL can express to some extent in the liver under chronic hepatitis. Next, we observed CAHL subcellular localization in Huh-7 and MH-14 cells using an anti-CAHL antibody (Fig. 3D). Fluorescence derived from CAHL

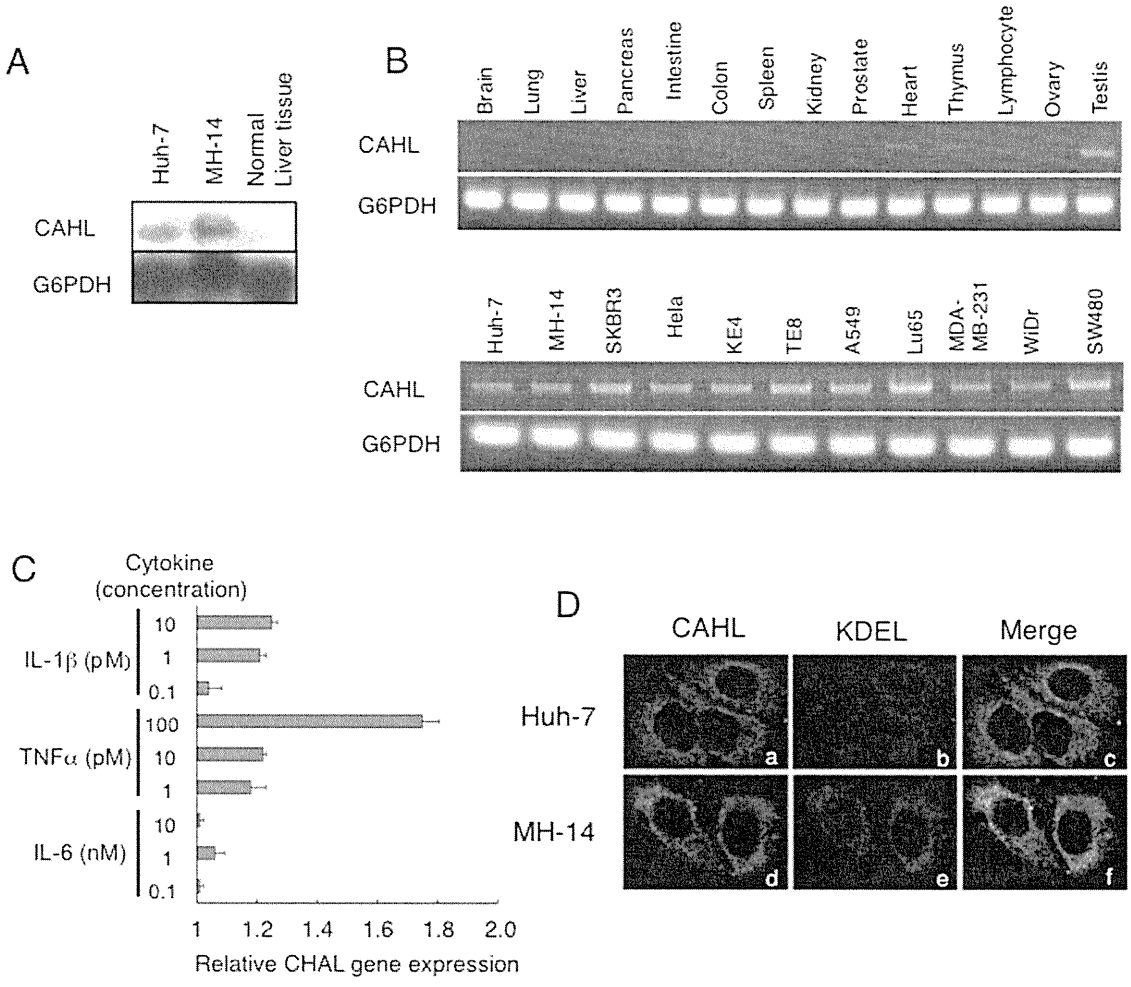

Figure 3. Expression profile of CAHL. (A) For Northern blot analysis, CAHL and G6PDH as an internal control were detected in RNAs derived from Huh-7 and MH-14 cells, as well as normal liver tissues. (B) RT-PCR analysis for CAHL, and G6PDH as an internal control, were performed using RNAs derived from normal human tissues and tumor cells. (C) CAHL in normal liver cells was upregulated by TNF-α. Normal liver cells were cultured in the presence of proinflammatory cytokines, IL-1β, TNF-α, and IL-6 at the indicated concentrations for 8 h. Subsequently, cells were harvested, and measurement of these cells derived-CAHL gene expression by quantitative analysis by was performed using the LightCycler system. These data represent as relative rates (1 = non-treated cells). Error bars represent the standard error of the mean. (D) Colocalization of CAHL with KDEL as an endoplasmic reticulum (ER) marker. Indirect immunofluorescence analysis was performed on Huh-7 and MH-14 cells. Cells stained with anti-CAHL (panels a and d, green) and an anti-KDEL mAb (panels b and e, red) for ER identification as a marker used as a primary antibody followed by Alexa Fluor 488-conjugated goat anti-rabbit and 594-conjugated goat anti-mouse antibodies, respectively. Merged images of green and red signals are shown in panels c and f.The optically merged image is representative of most cells examined by laser confocal microscopy. Original magnification: x1000.
doi:10.1371/journal.pone.0018285.g003

demonstrated that CAHL was co-localized with KDEL protein as a marker for endoplasmic reticulum (ER) in both the presence (MH-14 cells) and absence (Huh-7 cells) of the HCV subgenome, indicating that CAHL could localize in ER with HCV independent manner. Moreover, it was observed that CAHL also colocalized with HCV-derived proteins such as NS3, NS4A, NS4B, NS5A and NS5B localized in ER (Fig. S2). Thus, these data strongly suggested that CAHL would localize in ER.

Association of CAHL, NS5B and CyPB

Since CAHL interacts CsA, which has inhibitory effects to HCV replication, it is interesting to investigate the molecular interactions of CAHL and HCV-derived molecules involving the replication machinery. Intriguingly, the purified full-length CAHL fused to GST was coprecipitated with NS5B but not NS3, NS4B or NS5A protein, as shown in Fig. 4A. To determine a regions of NS5B responsible for binding with CAHL, various dissected NS5B proteins were subjected to pull-down assays, resulting in that two separated regions (1–200 aa or 401–520 aa) of NS5B were sufficient for the interaction with CAHL (Fig. 4B). In addition to the CAHL and NS5B interaction, we found interaction between CAHL and CyPB, but not CyPA (Fig. 4C). The interaction of CAHL and CyPB was disrupted with presence of CsA, whereas the association of CAHL with NS5B was not (Fig. 4D). These results suggest that trimer complex consisting of CAHL, CyPB and NS5B could form.

CAHL has a main role in HCV-replication via NS5B

The finding that CAHL structurally associated with the CyPB/NS5B complex in cell-free assessment prompted us to examine whether this trimer complex could act for HCV genome replication *in vivo*. First, five small interference RNAs (siRNA)

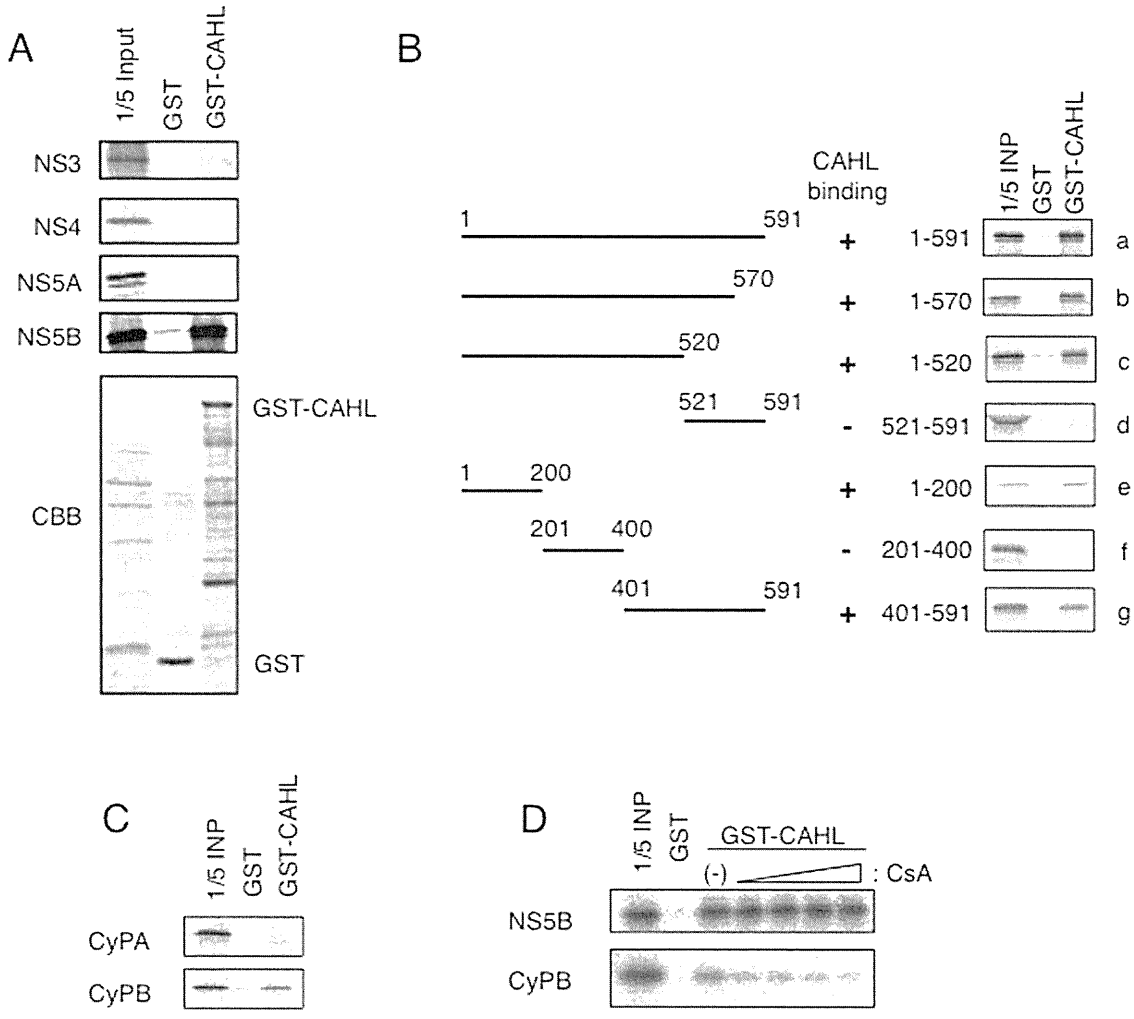

Figure 4. CAHL interacted with HCV NS5B and CyPB. (A) [^{35}S]-labeled in vitro translation products of HCV NS3, NS4B, NS5A, and NS5B were incubated with a recombinant GST fusion protein of CAHL (GST-CAHL) or GST as a negative control. "1/5 input" designates the signal for 1/5 the amount of the [^{35}S]-labeled product used in the pull-down assay. CBB staining patterns for the pulled-down proteins are shown in the bottom panel. (B) Mapping of the regions of NS5B responsible for the interaction with CAHL. At the left of the panel, schematic representations of the full-length and truncated mutants of NS5B are shown. The numbers indicate the amino acid residue numbers in NS5B. "CAHL binding" summarizes the results of the GST pull-down assay by +/−. GST pull-down data are presented as described in (A). (C) GST pull-down assay between GST-CAHL and in vitro translated CyPA or CyPB was performed as described in (A). (D) The interaction of CAHL with CyPB was disrupted by CsA treatment. GST pull-down assay between GST-CAHL and NS5B was performed in the absence and presence of CsA. The concentrations of CsA in lanes 4–7 are 1, 2, 8, and 20 μg/ml, respectively.

doi:10.1371/journal.pone.0018285.g004

specific for the CAHL gene (si-1, -2, -3, -4, and -5) were individually transfected into MH-14 cells to examine RNA sequence induced effectively down-regulation. When si-3 siRNA was transfected into cells, the endogenous CAHL gene expression reduced approximately 90% compared with si-control (treatment with siRNA for non-target gene) (Fig. S3), and among them, si-3 induced down-regulation of CAHL gene expression most effectively. Subsequently, we applied short hairpin RNA (shRNA) technology to stably knockdown CAHL gene expression in MH14 cells. We cloned DNA oligo coding the effective siRNA against CAHL gene into pLKO.1-puro shRNA vector. Lentivirus packed with shRNA against CAHL (sh-CAHL) or non-targeting shRNA (sh-control) were introduced into MH14 cells, and then these cells were cultured in the presence puromycin. As a result, we successfully obtained stably CAHL gene knockdown cell line, which reduced approximately to 6-fold compared with sh-control (Fig. 5A). In these sh-CAHL cells, HCV RNA was decreased approximately to 4-fold less than that in the sh-control cells (Fig. 5B). Furthermore, ectopic expression of CAHL increased the HCV replication level in a dose-dependent manner (Fig. 5C). These results suggest that CAHL positively plays in HCV replication.

To investigate the outcome of the interaction of CAHL with NS5B/CyPB, we performed RNA binding activity assay using sh-CAHL cells. NS5B is a viral RNA-dependent RNA polymerase and possesses RNA binding activity [11]. Indeed, NS5B formed a complex on RNA-immobilized sepharose together with CyPB and CAHL (lane 4 in Fig. 6A). However, shRNA-mediated depletion of endogenous CAHL dissociated CyPB from the NS5B/RNA complex (lane 6 in Fig. 6A), indicating that CAHL mediates the association of CyPB with NS5B/RNA. Moreover, when CsA was added to sh-CAHL cells, both CAHL and CyPB were dissociated from RNA (lane 6 in Fig. 6B). Thus, the possibility is suggested that the promotion of CyPB-NS5B complex association by CAHL is related with the stimulatory role of CAHL in HCV replication.

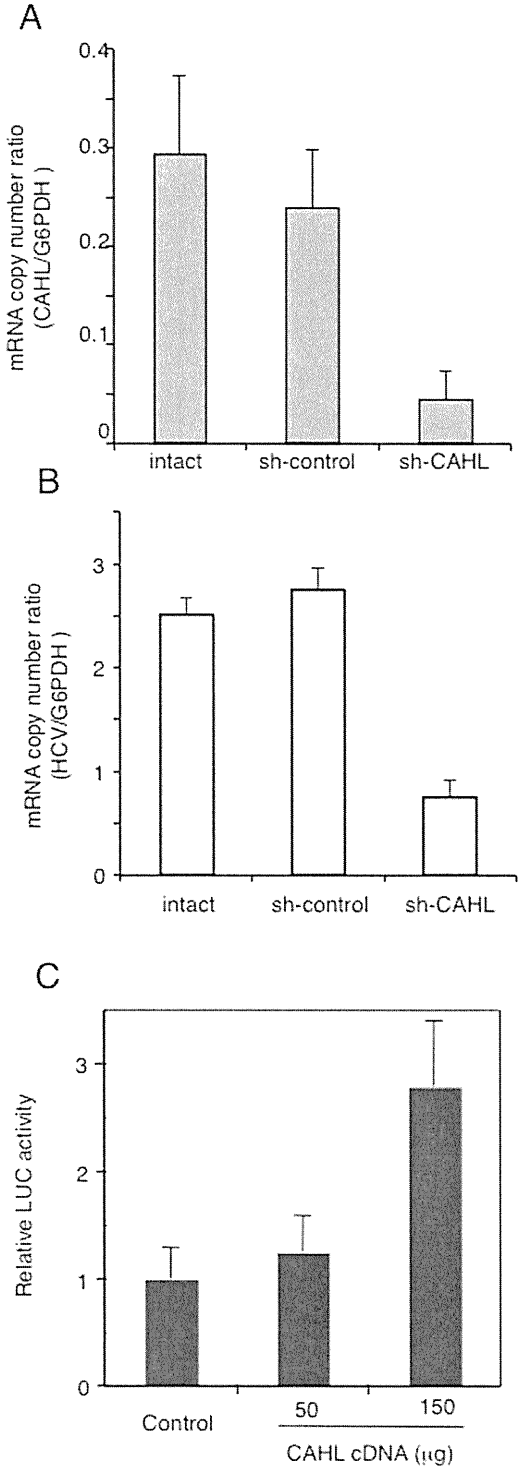

Figure 5. Establishment stably CAHL knockdown cell. (A) Lentivirus packed with shRNA against CAHL (sh-CAHL) or non-targeting shRNA (sh-control) were introduced into MH14 cells. Total RNAs were harvested and the absolute mRNA copy numbers of CAHL were examined by quantitative real time RT-PCR. (B) The same samples of total RNAs were used for the measurement of the absolute RNA copy number of the HCV genome by quantitative real time RT-PCR. (C) Cured MH-14 cells were transfected with LMH14 RNA reporter together with the expression plasmid for CAHL or the corresponding empty vector. At four days post-transfection, luciferase activities were measured. These results (A–C) represent the means of three independent experiments.
doi:10.1371/journal.pone.0018285.g005

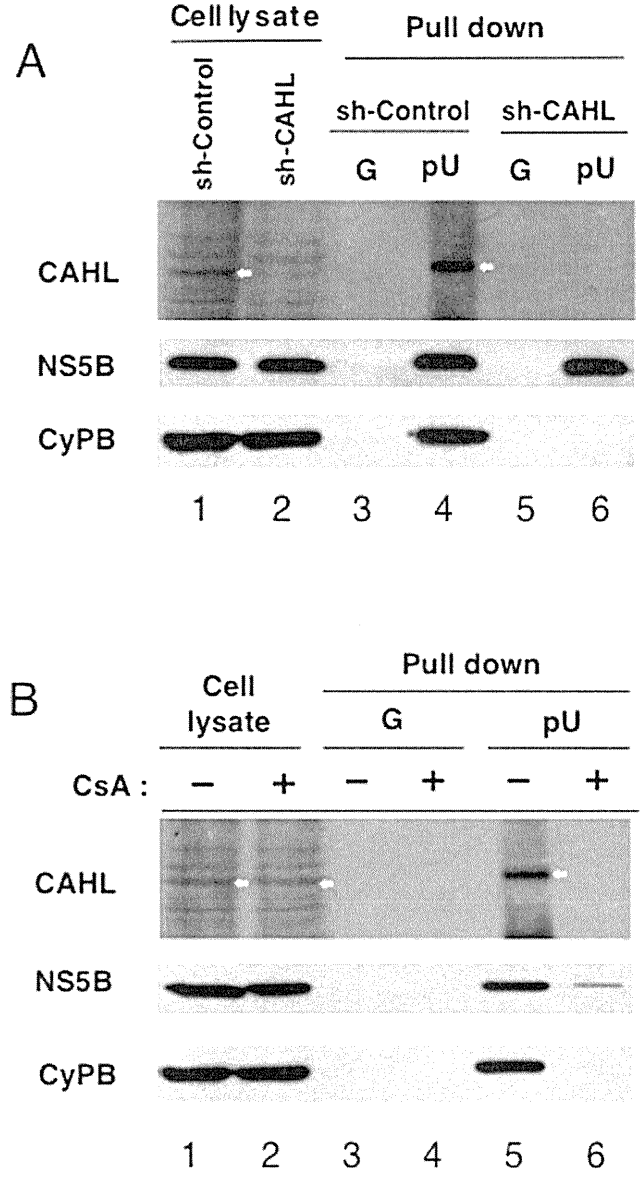

Figure 6. CAHL associated with the CyPB/NS5B complex plays critical roles in HCV replication. (A) Cells (sh-control or sh-CAHL cells) were harvested and analyzed protein expressions by using anti-CAHL, anti-NS5B, and anti-CyPB antibodies. White arrows indicate CAHL protein. (B) Cells were treated with or without 2 μg/ml CsA for 24 h, and then harvested and analyzed. These results were reproduced in three independent experiments. White arrows indicate CAHL protein.
doi:10.1371/journal.pone.0018285.g006

Discussion

Phage display, invented by Smith and Petrenko, is a versatile method for the detection of small molecule-binding proteins [14]. The technique can also be used to identify binding sites within the target protein itself. The combination of screening a library of phage-displayed peptides and analysis of affinity-selected peptides is anticipated to become a powerful tool for identifying drug-binding sites [15–19]. Screening phage display libraries generally entails immobilizing the drug onto a solid surface [20]. In the conventional method of phage display, small molecules should be converted into biotinylated derivatives and immobilized on a streptavidin-coated matrix. Conventional immobilization requires

the presence of desirable functional groups within the drug molecule as well as a multistep process to prepare the biotinylated derivatives. In contrast to biotinylation, photoimmobilization makes it possible to covalently immobilize drugs on a solid surface without the need for derivatization. We and Kanoh *et al.* have reported the affinity purification of proteins using affinity matrices, in which small molecules are photoimmobilized by photoreaction [12,21]. Because the photoreaction proceeds in a functional group-independent manner, the molecules are immobilized onto the solid surface in a nonoriented fashion. Thus, photoimmobilization can be a useful tool for the comprehensive analysis of drug-binding proteins.

Using this method, we identified CAHL as a novel target protein for CsA. CsA is a natural compound showing multiple biological activities, including an immunosuppressive function, anti-chaperone activity, inhibition of transporter activity and antiviral activity against human immunodeficiency virus and HCV. Thus far, p-glycoprotein and formyl peptide receptor, as well as CyPs, were reported as binding proteins for CsA [22], which enabled elucidation of the mechanism of the CsA-induced immunosuppressive function, anti-chaperone activity and anti-transporter activity, respectively. Although CyPA promotes HCV replication [11,23,24], we cannot fully explain the whole mode of action of CsA against HCV. CyPB is also reported to regulate HCV replication. It was reported that the HCV replicon showing resistance against the CsA-mediated anti-HCV effect possessed mutations in the coding region for NS5A and NS5B [25,26], indicating that NS5B was one of the determinants for the sensitivity to CsA. However, some such mutations within the NS5B coding region were dropped outside the region interacting with CyPA and CyPB [11,24], leading to the possibility that another cellular protein which is targeted by CsA, binds to NS5B and regulates HCV replication. The CAHL-NS5B regulation machinery is consistent with this idea. Deletion analysis for NS5B demonstrated that two separate regions (1-200aa and 401-520aa) of NS5B are likely to be involved in the interaction with CAHL. These regions are different from the NS5B domain interacting with CyPB (521-591aa) [11], suggesting that NS5B would interact with both CyPB and CAHL at the same time. Indeed, the mutations that induced resistant to CsA, the I432V in NS5B reside inside the regions interacting with CAHL (1-200 aa and 401-520 aa) [26], supported the relevance of CAHL in HCV genome replication. As another aspect, it is interesting that two CsA target molecules interact with each other and NS5B. Although we do not know in detail the implication of the interaction of these two CsA target molecules, CyPB and CAHL, there is a similar example already known: two FK506-binding proteins, P-glycoprotein (P-gp) and FKBP42, associate with each other [27]. In this situation, FKBP42 modulates P-gp function. We do not know in detail how these two target molecules of CsA, CyPB and CAHL both regulate NS5B function, which is a future subject of the study. Currently, a CsA derivative shows remarkable anti-HCV effect in chronic HCV-infected patients in the phase II clinical trial, and its mode of action needs to be fully clarified [28]. Our data suggest a new link of CAHL, in addition to CyP family, with CsA derivative's anti-HCV activity.

Cellular RNA helicases have been reported to be involved in HCV genome replication. DDX3 and DDX6 activate HCV genome replication through yet unknown mechanism [29,30]. RNA helicase p68 (DDX5) interacts with NS5B and supports HCV genome replication in a transient transfection assay [31]. Although the mechanism through which each RNA helicase regulates HCV genome replication may be different, the requirement of cellular RNA helicases for HCV genome replication is interesting for understanding HCV-cellular factors interaction.

CAHL expression in normal liver cells was much less than that in HCV infectious cells such as Huh-7 and MH-14. This is enigmatic since it is not clear how HCV replication start without CAHL, which positively plays HCV replication, at very beginning of HCV infection in normal liver cells. It was reported that a proinflammatory cytokine, TNF-α gene expression in hepatocytes and mononuclear cells derived from HCV carrier increased compared with healthy control [32]. As we here demonstrated CAHL induced by TNF-α, CAHL can express to some extent in the liver under chronic hepatitis C. We also show the association of CAHL with HCV replication. Taken together, CAHL may form a positive feedback loop for HCV replication: CAHL gene expression is induced by TNF-α that is highly upregulated by HCV infection, and CAHL in turn promotes HCV replication. Despite the low expression of CAHL in normal tissues, CAHL may have strong potential as a pharmaceutical target protein. In addition to CsA, isolation of specific inhibitors to the interaction of CAHL and NS5B could allow us to provide effective drug for HCV treatment.

In conclusion, we took advantage of strategy of chemical biology to isolate a cellular factor, CAHL, as CsA associated helicase-like protein, which would form trimer complex with CyPB and NS5B of HCV. These findings not only shed a light on new HCV treatment but also brought about great values of chemical biology to elucidate biological mechanisms of small-molecule and protein interactions.

Materials and Methods

Preparation of CsA-immobilized resins

CsA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CsA-immobilized resins were prepared on photoaffinity resins as described previously [12]. Photoaffinity resins treated with UV irradiation in the absence of CsA were used for negative control.

Phage display screening

10 mg of CsA-immobilized resin was incubated in 1 ml of TBS (50 μM Tris-HCl [pH 8.0] and 150 mM NaCl) for 12 hours or longer before use. Phage screening conditions were performed as previously described [20]. For each panning step, 50 μl of CsA-immobilized resin slurry was added to 1 ml of the T7 phage ($>10^{11}$ pfu) followed by incubation for 8 hours at 4°C. After incubation, the bead slurry was washed 10 times by adding 1 ml of TBST (50 mM Tris-HCl [pH = 8.0], 150 mM NaCl, 0.1% Tween 20). To elute phage particles associated with resins, 100 μl of *Escherichia coli* ($OD_{600} = 0.6$) was added, and incubated for 10 min at 37°C. The phage infected *E. coli* were transferred into 1 ml of *E. coli* ($OD_{600} = 0.6$) and grown until lysed for three hours at 37°C with shaking. For titer check, 10 μl of infected *E. coli* was used.

RNA preparation and plasmid construction

To isolate the CAHL gene, we used total RNA derived from human liver. DNA cloning of the CAHL gene was carried out using a SMART-cDNA isolation kit following the manufacturer's instructions (Clontech Laboratories, CA, USA). In some cases, CAHL cDNA was reconstructed with pcDNA 3.1 myc-HisA (Invitrogen Corp., CA, USA) for overexpression experiments.

Surface plasmon resonance assay

SPR analysis was performed on a BIAcore 3000 (Biacore AB, Uppsala, Sweden). The bacterially expressed CAHL-C was

immobilized covalently on a hydrophilic carboxymethylated dextran matrix on a CM5 sensor chip (Biacore AB) using a standard amine coupling reaction in 10 mM CH_3COONa [pH = 4.0]. Binding analyses were carried out in HBS-EP buffer (10 mM HEPES [pH = 7.4], 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) containing 8% DMSO at a flow rate of 20 µl/min. Appropriate concentrations of CsA were injected over the flow cell. CyPA or CyPB was not used as a positive control because of two reasons: 1) those CyPs can be used for a positive control as CsA-CyP binding, but not for CsA-CAHL binding, and 2) FK506 doesn't bind CyPs, so that it is difficult to compare association behaviors between FK506 and CsA. The bulk effects of DMSO were subtracted using reference surfaces. To derive binding constants, data were analyzed by means of global fitting using BIAevaluation version 3.1 (Biacore AB).

Preparation of recombinant protein

CAHL-C cDNA encoding C-terminal 761 to1430 amino acids was constructed into pET21a prokaryotic expression vector (Merck, Darmstadt, Germany), which has a His-tag. pET21-CAHL-C construct was transformed into *E.coli* BL21(DE3) strain. After overnight induction with 0.1 mM IPTG at 20°C, recombinant CAHL-C was purified by nickel column chromatography with HisTrap (Amersham biosciences, Uppsala, Sweden) according to a manufacturer's procedure. To concentrate and exchange the buffer, purified CAHL-C was concentrated up to 40 times with PBS by using Amicon Ultra 30 (Millipore, EMD, Germany).

ATPase assay

ATPase activity was measured as described by Okanami *et al.* [33]. Briefly, CAHL-C protein (500 ng) was incubated in 50 µl of helicase/ATPase buffer containing 1 µl of [γ-^{32}P]ATP (1 Ci/µmol) in the presence or absence of 100 ng of total RNA derived from liver at 30°C for 30, 60, and 180 min. An aliquot (10 µl) was removed at the appropriate time and added to 200 ml of a solution containing 50 mM HCl, 5 mM H_3PO_4 and 7% activated charcoal. After the charcoal was precipitated by centrifugation to remove unreacted ATP, 10 µl of the supernatant was subjected to Cerencov counting to quantitate released [^{32}P]phosphate.

Northern blot analysis and reverse transcription PCR (RT-PCR) analysis

Tumor cell-derived total RNA was prepared using an RNeasy Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's instructions and then reverse-transcribed to cDNA with Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mnnheim, Germany). A reverse-transcribed single strand DNA library of normal tissues was purchased from Clontec, Inc. (CA, USA). In Northern blot analysis, RNA samples were loaded to formaldehyde agarose gels and transferred onto a Hybond N membrane (GE Healthcare UK Ltd., Buckinghamshire, England). After UV-crosslinking, the membrane was hybridized with ^{32}P-labeled (Rediprime II, GE Healthcare) gene-specific probe, regions of $CAHL_{2913\text{-}4431}$ and human $G6PDH_{454\text{-}2016}$ and exposed to film for autoradiography. Measurement of CAHL gene expression by polymerase chain reaction (PCR) was performed using GoTaq Flexi DNA Polymerase (Promega, Co. WI, U.S.A.) and primer sets: forward primer, 5′-GACGGGAAAGGATTGGTCAA-3′ and reverse primer, 5′-CATCACTTCGTGCTTTTT-3′ for detection of CAHL, and forward primer, 5′-GACGAAGCGCAGACAGCGTCATGGCA-3′ and reverse primer, 5′-GCTTGTGGGGGTTCACCCACTTG-3′ for detection of G6PDH.

Quantitative real-time RT-PCR analysis

Total RNAs reverse-transcribed to cDNA were prepared as described above. Measurement of gene expression by quantitative analysis was performed using the LightCycler system (Roche Applied Science). Primers and hybridization probes were synthesized by Nihon Gene Research Laboratory Inc. (Sendai, Japan). Quantitative real time RT-PCR analyses of human glucose-6-phosphate dehydrogenase (G6PDH) and cyclosporin A associated helicase-like protein (CAHL, NM_022828) gene expression were performed using the LightCycler® FastStart DNA MasterPLUS SYBR Green I system (Roche Applied Science) with the following primer sets: forward primer, 5′-CTGCGTTATCCTCACCTTC-3′ and reverse primer, 5′-CGGACGTCATCTGAGTTG-3′ for detection of human G6PDH; forward primer, 5′-GTGT-CTGGACCCCATCCTTA-3′and reverse primer, 5′-CCCAT-CACTTCGTGCTTTTT-3′for detection of CAHL. Gene expression analysis of the HCV genome was performed using the LightCycler® FastStart DNA Master HybProbe system (Roche Applied Science) with the following primer set and probe: forward primer, 5′-CGGGAGAGCCATAGTGG-3′ and reverse primer, 5′-AGTACCACAAGGCCTTTCG-3′, and the fluorogenic probe, 5′-CTGCGGAACCGGTGAGTACAC-3′. PCR amplification of the housekeeping gene, G6PDH, was performed for each sample as control for sample loading and to allow normalization among samples. To determine the absolute copy number of the target transcripts, the fragments of G6PDH or target genes amplified by PCR using the above described primer set were constructed with pCR4®-TOPO® cloning vector (Invitrogen). The concentrations of these purified plasmids were measured by absorbance at 260 nm and copy numbers were calculated from concentration of samples. A standard curve was created by plotting the threshold cycle (Ct) versus the known copy number for each plasmid template in the dilutions. The copy numbers for all unknown samples were determined according to the standard curve using LightCycler version 3.5.3 (Roche Applied Science). To correct for differences in both RNA quality and quantity between samples, each target gene was first normalized by dividing the copy number of the target by the copy number of G6PDH, so that the mRNA copy number of the target was the copy number per the copy number of G6PDH. The initial value was also corrected for the amount of G6PDH indicated as 100% to evaluate the sequential alteration of the mRNA expression level.

Cell culture and transfection of siRNA and cDNA

The human tumor cell lines of breast adenocarcinoma MDA-MB-231, lung adenocarcinoma A-549, colon adenocarcinoma WiDr, hepatocellular carcinoma Huh-7, breast cancer SKBR3, cervical carcinoma HeLa, esophagus cancer KE-4, colon adenocarcinoma SW480, lung cancer Lu65, and esophagus squamous cell carcinoma TE-8 were obtained from Health Science Research Resources Bank (Sendai, Japan). These cells were cultured in Dulbecco's modified Eagle's medium (Huh-7, SKBR3, HeLa, KE-4, and SW480 cells), RPMI 1640 (A-549, WiDr, TE-8, and Lu65 cells) (SIGMA-ALDRICH, MO, USA), and Leibovitz's L15 (MDA-MB-231 cells) (Invitrogen) supplemented with 10% fetal bovine serum, MEM nonessential amino acids (Invitrogen), 200 unit/ml penicillin (Invitrogen), 200 µg/ml streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen). MH-14 cells carrying the HCV subgenomic replicon [34] were cultured in the DMEM medium supplemented with 10% fetal bovine serum, MEM nonessential amino acids (Invitrogen), 200 unit/ml penicillin (Invitrogen), 200 µg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen) and 300 µg/ml G418 (Invitrogen). Five small interfering RNA (siRNA) duplexes containing 3′dTdT over

the hanging sequence were synthesized (Sigma-Aldrich, St. Louis, MO). These sequences were: si-1; 5′-GGACAUUCGCAUUGAUGAG-3′, si-2; 5′-CCUGUAAUUUGACUCAUAA-3′, si-3; 5′- GCCUUGGAUGUAAAUCUCUUU -3′, si-4; 5′- GGAGCUUUCAGUGACCAUA -3′, si-5; 5′-GGUCAAAUAAUAGUAGGAA-3′. A non-targeting siRNA (Sigma-Aldrich) was used as control. Plasmid and siRNA transfection was performed described previously [35]. In siRNA study, total RNAs from transfected cells were harvested after transfection for 5 days and examined mRNA copy number of CAHL by quantitative real-time RT-PCR.

Establishment of stable CAHL-knockdown cell by shRNA

Based on the siRNA data, we applied short hairpin RNA (shRNA) technology platform (Sigma Mission*RNAi*) to stably knockdown CAHL gene expression in MH14 cells. DNA oligo coding the effective siRNAs against each MH14-CAHL gene (5′-CCGGGCCTTGGATGTAAATCTCTTTCTCGAGAAAGAGATTTACATCCAAGGCTTTTTTG -3′) (sh-CAHL) was cloned into pLKO.1-puro shRNA vector. Plasmid DNA including non-targeting shRNA as control (sh-control) was transfected into MH14 cells along with Lentiviral Packaging Mix consisting of an envelope and packaging vector (Sigma-Aldrich) to produce lentivirus packed with shRNA cassettes using the standard procedure. After transfection, cells were cultured in the presence of 10 μg/ml puromycin.

Indirect immunofluorescence analysis

Anti-CAHL polyclonal antibody serum was generated in rabbits immunized with $CAHL_{1237\text{-}1251}$, ILHPKRGTEDRSDQS, according to our lab protocol [35]. Anti-NS3, NS4B, and NS5B, and NS5A were kindly provided from Dr. Kohara at The Tokyo Metropolitan Institute of Medical Science, Japan and Dr. Takamizawa at Osaka University, Japan, respectively. Cells were fixed with ice-cold acetone for 1 min, and then stained with anti-CAHL and anti-KDEL mAb (Santa Cruz Biotechnology, CA, USA) for ER antibodies followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG and 594-conjugated goat anti-mouse IgG (Invitrogen), respectively, and visualized using a Bio-Rad MRC1024ES laser confocal scanning microscopy system (Bio-Rad Laboratories, CA, USA).

Immunoblot analysis

Immunoblot analysis was performed essentially as described previously [11,36].

RNA-protein binding precipitation assay

RNA-protein binding precipitation assay was essentially performed as described [11]. Briefly, to permeabilize plasma membrane, cells were treated 50 μg/ml digitonin (Nakarai Tesque Inc., Kyoto, Japan) in buffer B (20 mM HEPES-KOH [pH = 7.7], 110 mM KOAc, 2 mM MgOAc, 1 mM EGTA) at 25°C for 5 min. After treatment with 0.5 μg/ml proteinase K at 37°C for 5 min and washing with buffer B, cells were lysed in IP buffer (50 mM Tris-HCl [pH = 8], 150 mM NaCl, 0.5% NP-40, and protease inhibitory cocktail [Roche Applied Science]). After centrifugation, supernatants were incubated for 2 h with poly-U or protein G Sepharose resin (GE Healthcare). After four washes with IP buffer, precipitates were analyzed by immunoblot analysis. Supernatants after centrifugation were used as a positive control (designated Cell lysate in Fig. 4E and 4D).

GST-pull-down assay

GST-pull-down assay was performed as described previously [36].

Supporting Information

Figure S1 Predicted amino acid sequences of CAHL (NM_022828). Underlined residues (LLGQLRA) indicate identical sequence of phage clone #13.
(TIFF)

Figure S2 Indirect immunoflourescence analysis for colocalized with between CAHL and NS3, NS4A, NS4B, NS5A, and NS5B using Huh-7 (A) and MH-14 (B). The primary antibodies used were anti-CAHL (panels a, d, g, j, and m, green) and anti-NS proteins (panels b, e, h, k, and n, red) antibodies. Marge images of green and red signals are shown in panels c, f, i, l, and o.
(TIFF)

Figure S3 Determinant of knockdown efficiency against CAHL gene expression. Five siRNAs for the CAHL gene were individually transfected into MH-14 cells. After transfection, total RNAs of these cells were collected and examined mRNA copy number of CAHL by quantitative real-time RT-PCR.
(TIFF)

Table S1 List of phage clones and their encoding deduced peptide sequences screened by CsA biopanning. *Asterisk indicates the identical sequences.
(DOCX)

Author Contributions

Conceived and designed the experiments: KM HS KW K. Shimotohno SK K. Sakaguchi FS. Performed the experiments: KM HS KW KI TS KK KT HM AT. Analyzed the data: NS. Wrote the paper: KM HS KW FS.

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