Adenovirus vector-mediated assay system for hepatitis C virus replication

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ABSTRACT

The efficient delivery of the hepatitis C virus (HCV) RNA subgenomic replicon into cells is useful for basic and pharmaceutical studies. The adenovirus (Ad) vector is a convenient and efficient tool for the transduction of foreign genes into cells *in vitro* and *in vivo*. However, an Ad vector expressing the HCV replicon has never been developed. In the present study, we developed Ad vector containing an RNA polymerase (pol) I-dependent expression cassette and a tetracycline-controllable RNA pol I-dependent expression system. We prepared a hybrid promoter from the tetracycline-responsive element and the RNA pol I promoter. Ad vector particles coding the hybrid promoter-driven HCV replicon could be amplified, and interferon, an inhibitor of HCV replication, reduced HCV replication in cells transduced with the Ad vector coding HCV replicon. This is the first report of the development of an Ad vector-mediated HCV replicon system.

INTRODUCTION

Hepatitis C virus (HCV) is a member of *Flaviviridae* that contains a 9.6-kb positive-sense RNA genome. A total of 170-million people worldwide are infected with HCV, leading to chronic hepatic inflammation, hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma (1). Chronic infection with HCV is a major cause of hepatocellular carcinoma (1). Interferon (IFN) therapy is the gold standard method for HCV patients, but it is effective in only 50% of patients and its use has been limited because of severe side effects (2–4). Additional pharmaceutical therapies are needed to overcome HCV. However, the tropism of HCV is limited to chimpanzees and

humans, and the mechanism of HCV infection and replication is not fully understood. The HCV genome encodes a polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (5). An HCV subgenome replicon (called HCV replicon in the present study) consisting of a reporter gene and HCV NS genes has allowed various studies of HCV replication and the development of anti-HCV agents (6–8). The delivery of the HCV genome or HCV replicon is a powerful tool for basic and pharmaceutical research, and the transduction of *in vitro* translated HCV RNA genome is often performed by electroporation. However, a convenient and efficient method to transfer the 9.6-kb HCV RNA genome or the 8–9-kb HCV replicon has never been fully developed.

Transcribed RNAs are classified into rRNAs, mRNAs and short RNAs (tRNAs) in mammalian cells. RNA polymerases differ among the transcribed RNA species: RNA polymerase (pol) I for rRNAs, RNA pol II for mRNA and RNA pol III for short RNAs. RNA pol I transcribes RNA without a 5′-cap structure or a 3′-poly-A tail, and a plasmid vector encoding RNA pol I promoter and terminator has been applied to the development of RNA virus-expression system. For instance, influenza viruses, arenavirus and uukuniemi viruses are generated using RNA pol I-driven expression plasmid vectors coding each segment of negative-sense RNA (9–12). Recombinant adenovirus (Ad) vectors have been widely used to deliver foreign genes to a variety of cell types and tissues *in vitro* and *in vivo* in basic research and clinical therapy. Ad vector can be easily prepared, grown to a high titer, and used to efficiently transfer genes into dividing and non-dividing cells. Furthermore, several types of Ad vectors have been developed to expand their tropism and to increase the size of encoded genes (13,14). Ad vector encoding RNA pol I-driven expression of influenza virus

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RNA has been developed for the generation of vaccine seed strains and for basic influenza virus studies (15). These findings indicate that the RNA pol I Ad vector system can be a promising tool for basic and pharmaceutical studies on HCV. However, the development of an RNA pol I-driven vector system expressing the HCV RNA genome has never been reported.

In the present study, we developed an RNA pol I-driven vector system to monitor HCV replication using an HCV replicon in which structural genes were replaced by the luciferase gene. We prepared an Ad vector containing a tetracycline (tet)-regulated RNA pol I-expression cassette consisting of an RNA pol I-driven responsive vector and a *trans*-activator vector, and we successfully developed an Ad vector-mediated HCV replication system.

MATERIALS AND METHODS

Cell culture

Huh7.5.1 1bFeo [genotype 1b HCV replicon cell line, (8)] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and G418 (Nacalai Tesque, Kyoto, Japan) at $500\,\mu g/ml$. Huh7 and 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were maintained in a 5% CO_2 atmosphere at 37°C.

Preparation of RNA pol I-driven plasmid vectors

An RNA pol I expression-cassette was subcloned as follows: pHH21 (kindly provided by Dr Kawaoka, Tokyo, Japan) containing RNA pol I expression cassette was digested with *Afl*III, blunted by the Klenow fragment of DNA polymerase, ligated with *Eco*RI linker and digested with *Eco*RI/*Nhe*I, resulting in a fragment of the RNA pol I expression-cassette. The RNA pol I cassette was inserted into the *Eco*RI-*Xba*I site of pHM5 (16), generating pPol I. A fusion gene of enhanced green fluorescent protein and firefly luciferase (EGFPLuc, Clontech, Mountain View, CA, USA) was inserted into pPol I, resulting in pP$_I$WT-EL.

The subgenomic HCV sequence and the replication-incompetent subgenomic HCV sequence deleting GDD motif (MLVNGDDLVV) in NS5B were amplified by polymerase chain reaction (PCR) using pRepFeo as a template (8). The PCR fragments were inserted into pPol I, generating pPol I-1bFeo and pPol I-1bFeoΔGDD. The Feo fragment in pPol I-1bFeo or pPol I-1bFeoΔGDD was replaced with firefly luciferase, generating pPol I-HCV or pPol I-ΔGDD coding firefly luciferase reporter, HCV NS3, NS4A, NS5A and NS5B or mutated NS5B, respectively. A plasmid expressing β-galactosidase, pCMVβ, was purchased from Marker Gene Inc. (Eugene, OR, USA).

Preparation of tet-controllable RNA pol I-driven plasmid vectors

To develop the tet-controllable RNA pol I promoter expression system, the minimal cytomegalovirus promoter was replaced by fragments of RNA pol I promoters

(from −235 to −1, from −311 to −1 or from −412 to −1) in pHM5-TREL2 (17), generating pP$_I$235, pP$_I$311 or pP$_I$412. These RNA pol I plasmid vectors were used for optimization of the tetracycline responsive element (TRE)/RNA pol I chimeric promoter. pHM5-tTA, pHM5-rtTA and pHM5-TREL2 were used in tet-regulated experiments (17).

Preparation of Ad vector expressing HCV replicon

The HCV replicon fragments cloned from pPol I-HCV or pPol I-ΔGDD were inserted into pP$_I$235, and then the firefly luciferase was replaced by the renilla luciferase to form pP$_I$235-HCV or pP$_I$235-ΔGDD. Ad vectors were constructed by an improved *in vitro* ligation method (18). Briefly, pP$_I$235-EL, pP$_I$235-HCV and pP$_I$235-ΔGDD were digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I/PI-*Sce*I-digested pAdHM4 and pAdHM36, respectively. The resulting plasmids were digested with *Pac*I and transfected into 293 cells with SuperFect (Qiagen, Valencia, CA, USA). AdP$_I$235-EL, AdP$_I$235-HCV and AdP$_I$235-ΔGDD were purified by CsCl$_2$ gradient centrifugation and dialyzed with a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl$_2$ and 10% glycerol. The multiplicity of infection (MOI) of Ad vectors was measured using an Adeno-X rapid titer kit (Clontech). Ad-tTA vectors were prepared as previously described (17).

Expression of plasmid-based HCV replicon

Huh7 cells were transfected with $0.8\,\mu g$ of pPol I-HCV. After 24 h of incubation, the cells were lysed in LCβ (Toyo Ink, Tokyo, Japan). The cell lysates were frozen-thawed and centrifuged at 32 000 g for 5 min. The luciferase activity in the resulting supernatant was measured using a commercially available kit (PicaGene; Toyo Ink).

Inhibition assays of HCV replication in plasmid- or Ad-based RNA pol I HCV system

Huh7 cells were transfected with $0.8\,\mu g$ of pPol I-HCV and $0.2\,\mu g$ of pCMVβ or infected with AdP$_I$235-HCV (10 MOI) and Ad-tTA (50 MOI). After 2.5 or 1.5 h of transfection, the cells were treated with recombinant human interferon-$\alpha 8$ (IFN-$\alpha 8$) at the indicated concentration. After an additional 72 h of incubation, the cells were lysed in LCβ. Luciferase activity and β-galactosidase activity in the lysates was measured with PicaGene and a Luminescent β-gal Kit (Takara Bio Inc., Shiga, Japan), respectively. The cell viability was measured with a WST-8 kit according to the manufacturer's instruction (Nacalai Tesque).

Evaluation of tetracycline-controllable promoters in plasmid vector

Huh7 cells were co-transfected with $0.1\,\mu g$ of reporter plasmid (pP$_I$235-EL, pP$_I$311-EL, pP$_I$412-EL or pP$_I$WT-EL), $0.8\,\mu g$ of tet-responsive *trans*-activator plasmid (pHM5-rtTA in the tet-on system or pHM5-tTA in the tet-off system) and $0.1\,\mu g$ of pCMVβ. After 2.5 h, the cells were treated with doxycycline (Dox) at the indicated

concentration for 48 h. Then, luciferase and β-galactosidase activities in the lysates were measured.

Expression of Ad vector containing tetracycline-controllable promoter system

Huh7 cells were transfected with a reporter Ad vector (AdP$_I$235-EL or AdP$_I$235-HCV at MOI of 5 or 10) and a *trans*-activator vector (Ad-tTA at MOI of 10 and 50). After an additional 48 h of incubation, luciferase activity in the cell lysates was measured.

Western blotting

Huh7 cells were co-infected with AdP$_I$235-HCV at 10 MOI and Ad-tTA at 50 MOI. The cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA] containing a cocktail of protease inhibitors (Sigma, St Louis, MO, USA). The cell lysates (30 μg of protein) were subjected to sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE), followed by western blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking in 5% skim milk, the filter was incubated with mouse anti-NS5A (Meridian Life Science, Sacao, ME, USA) or anti-β-actin Ab (Sigma). Then, the peroxidase-labeled secondary antibodies were added. The immunoreactive bands were visualized by chemilumines-cence reagents (GE Healthcare, Buckinghamshire, UK).

Evaluation of NS5B-dependent replication

Huh7 cells were transfected with AdP$_I$235-HCV or AdP$_I$235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, luciferase activities in the lysates were measured.

Detection of a fragment of the HCV negative strand RNA

Huh7 cells were co-infected with AdP$_I$235-HCV or AdP$_I$235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. The total RNAs were purified with High Pure RNA Isolation kit (Roche, Mannheim, Germany). The RNAs were reverse-transcribed to cDNA using a commer-cial available kit [TaKaRa RNA PCR Kit (AMV) Ver. 3.0] and a primer for the HCV negative strand RNA (5′-GCCAGCCCCCGATTGGGG-3′) or a primer for GAPDH (5′-TCTACATGGCAACTGTGA-3′), respect-ively. The transcription products of NS3 and GAPDH were amplified by PCR using paired primers (5′-ATGG CGCCTATTACGGCC-3′ and 5′-TGGTCTACATTAG TGTAC-3′) and (5′-GGTGGTCTCCTCTGACTTCAA CA-3′ and 5′-GTGGTCGTTGAGGGCAATG-3′), re-spectively. The putative sizes of the PCR products were 242-bp for NS3 and 89-bp for GAPDH. The PCR products were separated on 2% agarose gel.

RESULTS

RNA pol I-driven plasmid vector

First, we constructed an RNA pol I-driven plasmid coding an HCV replicon in which structural coding genes were replaced by the luciferase gene (Figure 1A). To investigate the expression of the HCV replicon from the RNA pol I plasmid vector, we transfected the plasmid vector into Huh7 cells. As shown in Figure 1B, the luciferase activity was observed in the RNA pol I vector-transfected cells. IFN is the most popular agent used to inhibit HCV replication. To examine whether the RNA pol I plasmid vector functions as an assay system for anti-HCV activity, we investigated the effect of IFN on the expression of the HCV replicon in the RNA pol I plasmid-transfected Huh7 cells. IFN dose-dependently reduced the replication of the HCV genome (Figure 1C), reaching 29.2% of the control at 5 pg/ml. IFN treatment did not cause any cytotoxicity (Figure 1D). These data suggest that the RNA pol I plasmid coding the HCV replicon works as an assay system for HCV replication.

RNA pol I-driven Ad5 vector

The Ad vector is the most efficient gene transfer vector for a variety of mammalian cells *in vitro* and *in vivo* (13,14,19,20). There are more than 51 serotypes of Ad. The Ad type 5 (Ad5) vector has been frequently used in basic research and clinical studies (21). Ad5 vectors are 100- and 1000-fold more efficient at mediating gene transduction than cationic lipids, an effective non-viral vector (22). A reverse genetics system for the generation of influenza virus using RNA pol I-driven Ad5 vector produced 1000-fold the virus titer of the RNA pol I plasmid system (15). These findings indicate that the Ad5 vector may have advantages for the preparation of an HCV replicon system. We prepared RNA pol I-driven Ad5 vectors and confirmed the expression of a reporter gene from the Ad5 vectors coding luciferase (Supplementary Figure S1). However, we did not succeed in preparing Ad5 vector particles coding the HCV replicon. Indeed, there have been no previous reports of the preparation of Ad5 vector expressing the HCV RNA genome.

We think that two problems must be solved in order to develop Ad5 vectors coding the HCV RNA genome. These problems are the influence of the HCV replicon on the preparation of Ad5 particles and the packaging limit of Ad5 vectors.

Preparation of the TRE/RNA pol I chimeric promoter

The tet-regulated system comprises a regulator vector that expresses tet-controlled *trans*-activators and a response vector consisting of TRE within the promoter that controls expression of the gene of interest. The tet-controlled *trans*-activators are classified into tTA and rtTA that binds to the TRE promoter and activates ex-pression from the TRE promoter in the absence and presence of Dox, respectively (23,24). We speculated that a tet-regulated vector system would minimize the influence of the HCV replicon on the preparation of Ad vector
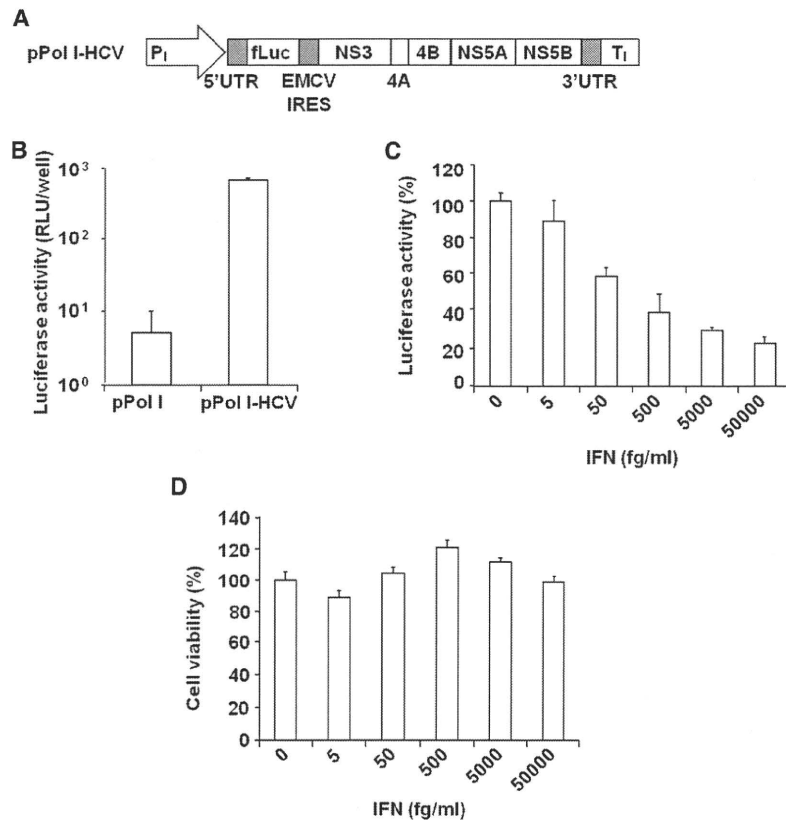
Figure 1. Preparation of plasmid expressing HCV replicon driven by RNA pol I promoter. (**A**) Schematic construct of HCV replicon-expression cassette. The HCV replicon gene was driven by the RNA pol I promoter (P_I) and terminator (T_I). (**B**) Transgene expression in Huh7 cells. Cells were transfected with pPol I-HCV. After 24 h of transfection, the luciferase activities were measured. Data are mean ± SD ($n = 3$). (**C** and **D**) Effect of IFN on HCV replication in RNA pol I vector-transfected cells. Huh7 cells were transfected with pPol I-HCV. After 2.5 h of transfection, the cells were treated with IFN at the indicated concentration. After an additional 72 h of incubation, the luciferase activity (C) and the cell viability (D) were measured. The luciferase activity (%) was calculated as a percentage of that in the vehicle-treated cells. Data are mean ± SD ($n = 3$).

particles. First, we optimized the chimeric promoter of TRE and the RNA pol I promoter. As shown in Figure 2A, the RNA pol I promoter is a 412-bp fragment containing an upstream control element (UCE) and the binding site of a transcription factor (Core). We constructed three chimeric promoter-driven plasmid vectors and checked the expression profiles using luciferase as a reporter gene. The chimeric vector was co-transfected into Huh7 cells with response vectors coding tTA or rtTA (23,24). As shown in Figure 2B and C, co-transfection with tTA exhibited a higher expression level than that of rtTA. The P_I235 promoter had the lowest luciferase expression in the absence of response vectors (Supplementary Figure S2). We used tTA and the P_I235 promoter in further studies. To investigate whether the chimeric RNA pol I promoter works in the Ad vector, we prepared Ad5 vector coding the chimeric RNA pol I-driven luciferase gene. AdP$_I$235-EL (MOI of 5) was co-transduced with Ad-tTA at MOI of 10 and 50. As shown in Figure 2D, the luciferase expression was increased in an Ad-tTA concentration-dependent manner.

Expression of the HCV replicon from Ad vector

The packaging limit of a foreign gene in the conventional Ad5 vector has been estimated to be 8.1–8.2-kb (25). The HCV replicon is ~8.9-kb and contains a 1.7-kb firefly luciferase gene and sequence derived from the HCV genome. Thus, another reason for no previous reports regarding the preparation of Ad5 vector coding the HCV replicon appears to be the packaging limit of the Ad5 vector. Mizuguchi and Hayakawa found that Ad5/35 vector containing chimeric fibers of Ad5 and Ad35 increased the size limit of foreign genes to 8.8-kb (26). We were successful in preparing Ad5/35 vector particles (9.53×10^8 IFU/ml) coding the TRE/RNA pol I chimeric promoter-driven HCV replicon containing the 1.0-kb renilla luciferase gene and sequence derived from the HCV genome (Figure 3A). To investigate the expression of the HCV replicon, Huh7 cells were transfected with the Ad vector coding the HCV replicon and Ad-tTA at MOI of 10 and 50, respectively. As shown in Figure 3B, western blot analysis showed that NS5A was expressed in Huh7 cells transfected with the vectors in the absence of Dox.
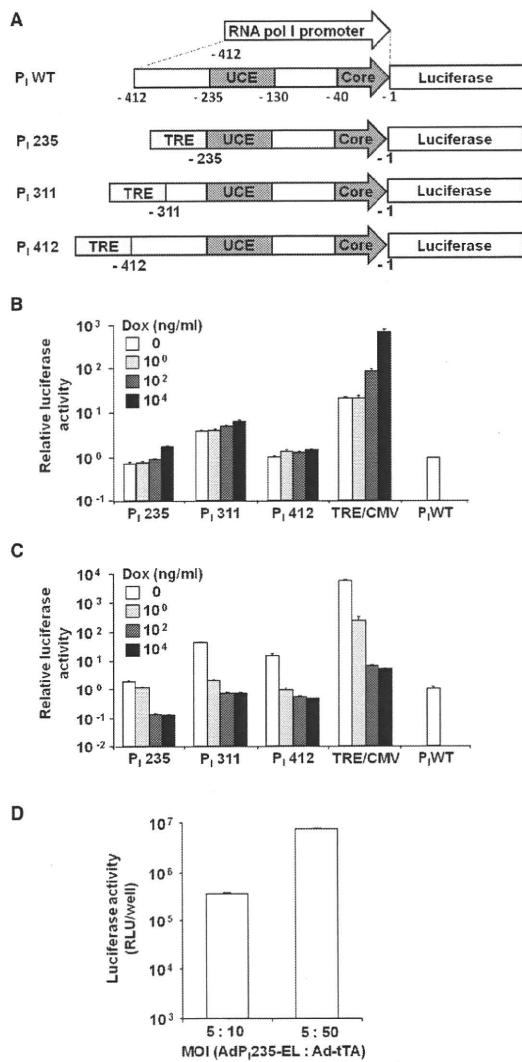
Figure 2. Development of tet-controllable RNA pol I promoter. (**A**) Construct of the chimeric RNA pol I promoter. The 412-bp human RNA pol I promoter contains Core (from −40 to −1), the binding site of the transcription factor, and UCE (from −235 to −130). TRE is connected to the full or partial RNA pol I promoter at the indicated sites, resulting in P$_I$235, P$_I$311 and P$_I$412. (**B** and **C**) Promoter activities of the chimeric promoter in Huh7 cells. Huh7 cells were co-transfected with the chimeric RNA pol I plasmid coding EGFPLuc, pCMVβ and *trans*-activator plasmid [rtTA (B) or tTA (C)]. After 2.5 h of transfection, the cells were treated with Dox at the indicated dose. After an additional 48 h of incubation, the luciferase and β-galactosidase activities were measured. The luciferase activity was normalized by the β-galactosidase activity and expressed relative to that of pP$_I$WT-EL-transfected cells. Data are mean ± SD (*n* = 3). (**D**) Transgene activity of Ad vector coding the chimeric promoter construct. Huh7 cells were co-infected with AdP$_I$235-EL and Ad-tTA. The MOI ratio of AdP$_I$235-EL to Ad-tTA was 5:10 or 5:50. After an additional 48 h of incubation, the luciferase activity was measured. Data are the mean ± SD (*n* = 3).

Luciferase was also expressed (Figure 3C). Dox dose-dependently attenuated expression of luciferase (Supplementary Figure S3). To discriminate between translation of the RNA pol I-transribed HCV RNA derived from the vector DNA and translation of HCV RNA derived from autonomous HCV replication in the transcribed cells, we prepared replication-incompetent HCV replicon deleting GDD motif in NS5B. Luciferase expression was attenuated in the cells transfected with the GDD-deleted Ad vector (AdP$_I$235-ΔGDD) (Figure 3D). A fragment of the HCV negative strand RNA, an essential replication intermediate, amplified by RT-PCR has been detected in the cells transfected with AdP$_I$235-HCV but not AdP$_I$235-ΔGDD (Figure 3E). Autonomous replication of the HCV RNA may occur in this system. To evaluate whether the Ad vector systems could be used to evaluate inhibitors of HCV replication, we investigated the effect of IFN on luciferase expression from HCV replicon in the Ad vector. As shown in Figure 3F, treatment of cells with 5 pg/ml of IFN reduced luciferase expression (33.3% of vehicle-treated cells). Cell viability was not affected by IFN treatment (Figure 3G). These findings indicate that the tet-controllable RNA pol I Ad vector may be useful for evaluation of anti-HCV activity.

DISCUSSION

HCV is an RNA virus containing the positive strand of a 9.6-kb RNA genome. A technique to transfer all or part of the HCV RNA genome to cells could be widely applicable for basic studies on HCV and pharmaceutical therapy against HCV. However, efficient and convenient methods to transduce the HCV RNA genome have never been fully developed. Electroporation of *in vitro* translated HCV RNA genome into cells is the most popular method. In the present study, we used a tet-controllable expression system to successfully develop an Ad vector system expressing the HCV RNA genome.

To our knowledge, development of Ad vector expressing HCV subgenome or genome has never been succeeded. The NS3 protease is essential for processing most of the NS proteins from the HCV polyprotein (27–30). The cleavage site of the NS3 protease is estimated to be between the P1 and P1′ position of an acidic amino acid at the P6 position, a Cys or Thr residue at the P1 position, and a Ser or Ala residue at the P1′ position (31). E1A, pIIIa, pol and V proteins of Ad have the cleavage site of the NS3 protease. The lack of previous success in generating Ad vectors coding the HCV genome and subgenome might be partly due to the degradation of Ad components by the NS3 protease during the preparation of Ad particles. In the tet-regulated system, when Ad vectors coding foreign genes driven by the TRE hybrid promoter are co-transfected with tTA or rtTA vector, the foreign gene can be expressed. Expression of the foreign gene could be suppressed during amplification of Ad vector particles in 293 cells, resulting in the preparation of Ad vector particles. The critical factor in the HCV replicon must be determined in a future study.
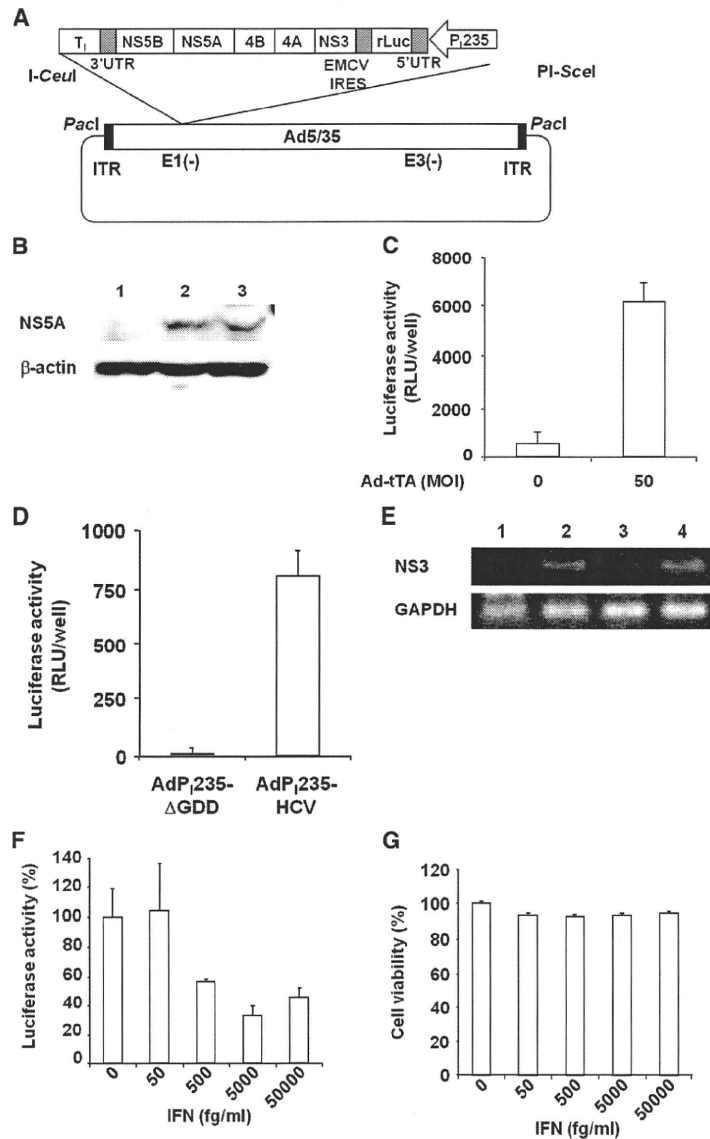
Figure 3. Preparation of Ad vector to monitor HCV replication. (**A**) Construct of Ad vector. The Ad vector contained the chimeric RNA pol I promoter ($P_I 235$) and the HCV replicon to monitor HCV replication as the luciferase expression. (**B**) Expression of HCV NS5A protein in Huh7 cells transfected with $AdP_I 235$-HCV. The cells were transfected with $AdP_I 235$-HCV (10 MOI) and Ad-tTA (50 MOI). After 72 h of incubation, the cells were harvested, and the lysates (30 μg) were subjected to SDS–PAGE, followed by immunoblotting with antibody against NS5A. Huh7 cells and Huh7.5.1 1bFeo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7 cells infected with $AdP_I 235$-HCV; lane 3, Huh7.5.1 1bFeo cells. (**C**) Expression of luciferase in the Ad vector-transfected cells. Huh7 cells were co-infected with $AdP_I 235$-HCV (10 MOI) and 0 or 50 MOI of Ad-tTA. After an additional 48 h of incubation, the luciferase activity was measured. Data represent the mean ± SD ($n = 3$). (**D**) Involvement of NS5B in expression of luciferase in the Ad vector-transfected cells. Huh7 cells were infected with $AdP_I 235$-HCV or $AdP_I 235$-ΔGDD (3 MOI) and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, the luciferase activity was measured. Data represent the mean ± SD ($n = 3$). (**E**) Expression of minus-stranded HCV RNA in the Ad vector-transfected cells. Huh7 cells were co-infected with $AdP_I 235$-HCV or $AdP_I 235$-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then RT-PCR analysis was performed for detection of minus-stranded HCV NS3 and GAPDH. The PCR products were separated on 2% agarose gel. Huh7 cells and Huh7.5.1 1bFeo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7.5.1 1bFeo cells; lane 3, Huh7 cells infected with $AdP_I 235$-ΔGDD; lane 4, Huh7 cells infected with $AdP_I 235$-HCV. (**F** and **G**) Effect of IFN on the replication of HCV replicon. Huh7 cells were infected with $AdP_I 235$-HCV (10 MOI) and Ad-rtTA (50 MOI). After 1.5 h of infection, the cells were treated with IFN at the indicated concentration for 72 h. Then, the luciferase activity (**F**) and the cell viability (**G**) were measured. Data represent the percentage of vehicle-treated cells. Data are the mean ± SD ($n = 3$).

Transgenes delivered by a conventional Ad5 vector are limited to a size of 8.1–8.2-kb (32), and the size of HCV replicon is ∼8.2-kb (containing a 1.0-kb luciferase gene and a 7.2-kb fragment of HCV genome) (8). The lack of a successful preparation of Ad5 vector may be partly due to limitation of packaging transgene. Mizuguchi and Hayakawa prepared a chimeric Ad vector containing type 5 and type 35 fiber proteins, which is a package 8.8-kb of foreign gene (26). CD46 is a receptor for Ad type 35 (Ad35), and CD46 is ubiquitously expressed in human cells (33,34). The Ad5/35 chimera vector can transduce various human cells more effectively than Ad5 vectors, indicating that the Ad5/35 vector may be a better system than Ad5 (26,35). In this study, we successfully prepared an Ad5/35 vector coding a tet-regulated RNA pol I-driven HCV replicon, and we found that the Ad5/35 vectors could be applied to evaluation of anti-HCV activity.

In conclusion, to the best of our knowledge, this is the first report to establish a novel strategy for the preparation of Ad vector expressing the HCV genome by using a tet-controllable expression system. Replication-incompetent HCV particles will be a promising candidate for vaccine therapy for HCV. As mentioned above, the packaging size (8.8-kb) of Ad5/35 vector used in the present study is smaller than that of the HCV RNA genome (9.6-kb), and, therefore, the preparation of inactive HCV particles using Ad5/35 vector is impossible. Helper-dependent Ad vector (HDAd), in which all viral coding sequences are deleted, can deliver a large capacity of ∼37-kb to cells (36). Tet-controllable RNA pol I HDAd vector might contribute to the development of vaccine therapy for HCV.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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PLoS one

A Novel Screening System for Claudin Binder Using Baculoviral Display

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Abstract

Recent progress in cell biology has provided new insight into the claudin (CL) family of integral membrane proteins, which contains more than 20 members, as a target for pharmaceutical therapy. Few ligands for CL have been identified because it is difficult to prepare CL in an intact form. In the present study, we developed a method to screen for CL binders by using the budded baculovirus (BV) display system. CL4-displaying BV interacted with a CL4 binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), but it did not interact with C-CPE that was mutated in its CL4-binding region. C-CPE did not interact with BV and CL1-displaying BV. We used CL4-displaying BV to select CL4-binding phage in a mixture of a scFv-phage and C-CPE-phage. The percentage of C-CPE-phage in the phage mixture increased from 16.7% before selection to 92% after selection, indicating that CL-displaying BV may be useful for the selection of CL binders. We prepared a C-CPE phage library by mutating the functional amino acids. We screened the library for CL4 binders by affinity to CL4-displaying BV, and we found that the novel CL4 binders modulated the tight-junction barrier. These findings indicate that the CL-displaying BV system may be a promising method to produce a novel CL binder and modulator.

Introduction

Tight junctions (TJ) are intercellular adhesion complexes in epithelial and endothelial cells; TJs are located in the most apical part of the complexes [1]. TJs have a barrier function and a fence function [2–4]. TJs contribute to epithelial and endothelial barrier functions by restricting the diffusion of solutes through the paracellular pathway. TJs maintain cellular polarity by preventing the free movement of membrane proteins between the apical and basal membranes [5]. Loss of cell-cell adhesion and cellular polarity commonly occurs in the early stages of cancer [6]. Modulation of the TJ barrier function can be a method to enhance drug absorption, and TJ components exposed on the surface of cancer cells can be a target for cancer therapy.

Biochemical analyses of TJs have identified TJ components, such as occludin, claudins (CLs) and junction adhesion molecule [7]. The CL family contains more than 20 integral tetra-transmembrane proteins that play pivotal roles in the TJ barrier and fence functions. CL1-deficient mice lack the epidermal barrier, while CL5-deficient mice lack the blood-brain barrier [8,9], indicating that the regulation of the TJ barrier by modulation of CLs may be a promising method for drug delivery. *Clostridium perfringens* enterotoxin (CPE) causes food poisoning in

humans [10]. An interaction between the C-terminal domain of CPE (C-CPE) with CL4 deregulates the TJ barrier [11,12]. We previously found that C-CPE enhances jejunal absorption through its interaction with CL4, indicating that a CL binder is a potent drug-delivery system [13].

The majority of lethal cancers are derived from epithelial tissues [14]. Malignant tumor cells frequently exhibit abnormal TJ function, followed by the deregulation of cellular polarity and intercellular contact, which is commonly observed in both advanced tumors and the early stages of carcinogenesis [6]. Some CLs are overexpressed in various types of cancers. For example, CL3 and CL4 are overexpressed in breast, prostate, ovarian, pancreatic and gastric cancers. CL1, CL7, CL10 and CL16 are overexpressed in colon, gastric, thyroid and ovarian cancers, respectively [15,16]. These findings indicate that the CLs may be a target molecule for cancer therapy. A receptor for CPE is CL4 [11,12]. CPE has anti-tumor activity against human pancreatic and ovarian cancers without side effects [17,18]. The CLs binders will be useful for cancer-targeting therapy.

As above, recent investigations of CLs provide new insight into their use as pharmaceutical agents; for example, a CL binder may be used in drug delivery and anti-tumor therapy. Selection of a CL binder by using a recombinant CL protein is a putative method to

prepare a CL binder. However, CLs are four-transmembrane proteins with high hydrophobicity; there has been little success in the preparation of intact CL protein. Recently, a novel type of protein expression system that uses baculovirus has been developed. Membrane proteins are displayed on the budded baculovirus (BV) in their active form [19–21], indicating that the BV system may be useful for the preparation of a CL binder. In the present study, we investigated whether a CL binder was screened by using a CL-displaying BV.

Results

Preparation of CL4-displaying BV

C-CPE is the only known CL binder and modulator [12,13,22]. C-CPE has affinity to CL4 in a nanomolar range [23]. We chose C-CPE and CL4 as models of the CL binder and CL, respectively. Several reports indicate that membrane proteins expressed on the surface of BV are in an intact form [19–21]. To check the expression of CL4 on the BV, we performed immunoblot analysis of the lysate of CL4-BV against CL4. As shown in Fig. 1A, CL4 was detected in the virus lysates. To determine if the CL4 expressed on the virus has an intact form, we performed enzyme-linked immunosorbent assay (ELISA) with CL4-BV-coated immunoplates. C-CPE binds to the extracellular loop domain of CL4 [23]. After the addition of C-CPE to the CL4-BV-coated plate, the C-CPE bound to the CL4-BV-coated plate was detected by anti-his-tag antibody, followed by incubation with horseradish peroxidase-labeled antibody. C-CPE was dose-dependently bound to CL4-BV, whereas C-CPE did not interact with wild-BV (Fig. 1B). Deletion of the CL4-binding region (C-CPE303) attenuated the interaction of C-CPE with CL4-BV (Fig. 1C). Together, these results indicate that the CL4 displayed on BV may have an intact extracellular loop region.

Selection of C-CPE-phage by using CL4-BV

We next examined the interaction between C-CPE-phage and CL4-BV. As shown in Fig. 2A, C-CPE-phage bound to CL4-BV but not to wild-BV, and a scFv-phage did not bind to CL4-BV. To determine if CL-BV can be used to select CL binders, we prepared a mixture of C-CPE-phage and scFv-phage at a ratio of 2:10 and used CL4-BV to select CL4-binding phage in the mixtures. The amount of C-CPE-phage was increased to 11 of 12 clones in the mixture (Fig. 2B), indicating that CL-BV may be useful in the preparation of CL binders.

We previously found that each substitution of S304, S305, S307, N309, S313 and K318 with alanine increased the binding of C-CPE to CL4 [24]. Here, we prepared a phage library for C-CPE by randomly changing the functional 6 amino acids to any of the 20 amino acids. To confirm the diversity of the library, we checked the sequences of 17 randomly isolated clones. Each of the 17 clones had a different sequence, indicating that the library has a diverse population of C-CPE mutants (Table 1).

Then, we screened the CL4-binding phage by their affinity to CL4-BV. After addition of the C-CPE library to CL4-BV-adsorbed tubes, the CL4-BV-bound phages were recovered (1st screening). We repeated this screening process two more times (2nd screening and 3rd screening). If the number of CL4-bound phage is increased during the screening, the ratio of the incubated phage titers to the recovered phage titers will increase. As shown in Fig. 3A, the ratio was increased during screening from 4.5×10^{-7} to 5.5×10^{-5}, indicating that the screening system for CL4 binders may work. Indeed, the number of monoclonal phage clones with high affinity to CL4-BV was increased after the 3rd screening compared with that after the 2nd screening (Fig. 3B).
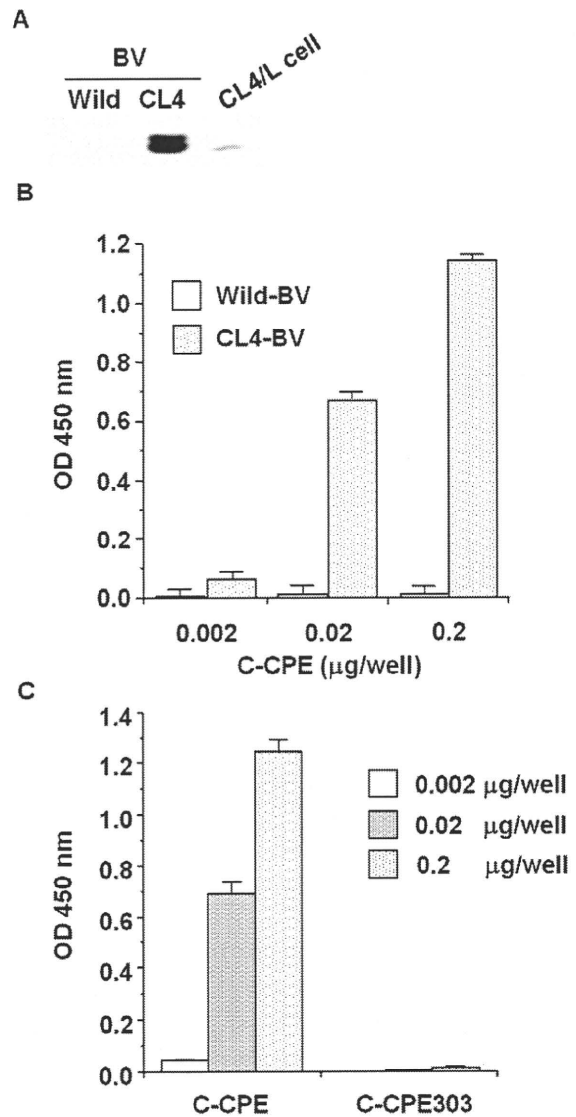

Figure 1. Preparation of CL4-displaying BV. A) Immunoblot analysis. Wild-BV and CL4-BV (0.1 µg/lane) were subjected to SDS-PAGE, followed by immunoblot analysis with anti-CL4 antibody. The lysate of CL4-expressing L (CL4/L) cells was used as a positive control. B, C) Interaction of a CL4 binder with CL4-BV. Immunotubes were coated with the wild-BV or CL4-BV, and C-CPE (B) or mutated C-CPE (C) was added to the BV-coated immunotubes at the indicated concentration. C-CPE bound to the BV-coated tubes was detected by ELISA with an anti-his-tag antibody.
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We analyzed the sequences of the CL4-BV-bound phages and got novel CL4-binder candidates with amino acid sequences that differed from the wild-type sequence (Table 2). To investigate their CL4-binding, we prepared the recombinant proteins of the binders and investigated their interaction with CL4 by ELISA with CL-BVs. As shown in Fig. 4A, the novel C-CPE derivatives had affinity to CL4 but not CL1. Next, we investigated whether the novel CL4 binders modulate TJ barrier in Caco-2 monolayer cell sheets, a popular model for the evaluation of TJ barriers [25].
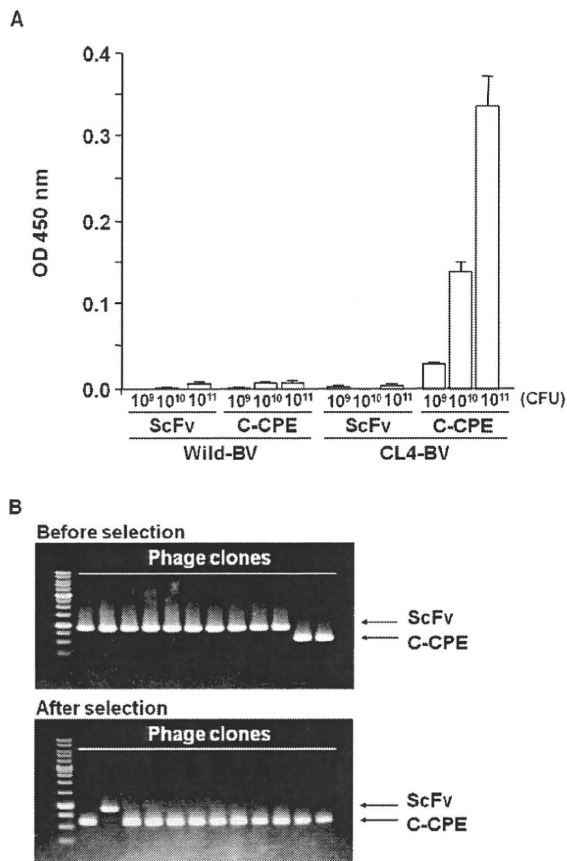
A

B

Before selection

After selection

Figure 2. Selection of C-CPE-displaying phage by using the CL4-BV system. A) Interaction of C-CPE-displaying phage with CL4-BV. Wild-BV or CL4-BV was coated on an immunoplate, and then scFv-displaying phage or C-CPE-displaying phage was added to the BV-coated immunoplate at the indicated concentrations. The BV-bound phages were detected by ELISA with anti-M13 antibody as described in Materials and methods. Data are representative of two independent experiments. Data are means ± SD (n = 3). B) Enrichment of C-CPE-displaying phage by the BV system. A mixture of scFv-phage and C-CPE-phage (mixing ratio of scFv-phage to C-CPE-phage = 2:10) was incubated with a CL4-BV-coated immunotube, and the bound phages were recovered. Each phage clone was identified by PCR amplification, followed by agarose gel electrophoresis. Upper and lower pictures are before and after the selection, respectively. The putative sizes of the PCR products are 856 and 523 bp in scFv and C-CPE, respectively. The data are representative of two independent experiments.
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Treatment of the cells with C-CPE resulted in decreased transepithelial electrical resistance (TEER) values, a marker of TJ integrity, and the TEER values increased after removal of C-CPE. The C-CPE derivatives (clones 1–5) had TJ-modulating activity similar to that of C-CPE (Fig. 4B).

Discussion

CL is a promising target for pharmaceutical therapy. However, CL has low antigenicity, and there has been little success in the preparation of monoclonal antibody against the extracellular loop region of CL. The three-dimensional structure of CL has never been determined, so it is impossible to perform a theoretical design

Table 1. C-CPE phage library.

C-CPE	304 S	305 S	307 S	309 N	313 S	318 K
Clone 1	V	T	C	V	N	K
2	C	P	A	H	L	T
3	A	G	G	V	P	P
4	R	G	H	L	E	H
5	A	A	P	S	R	Q
6	P	A	P	D	P	A
7	C	T	T	T	N	K
8	H	P	S	P	G	H
9	R	G	G	R	N	R
10	A	P	S	T	Q	P
11	V	L	G	N	M	R
12	P	P	A	T	F	R
13	G	D	C	S	N	L
14	F	R	V	F	R	N
15	S	Q	Q	W	T	T
16	S	R	L	E	W	Q
17	K	R	E	R	Q	S

Phage clones were randomly picked up from the C-CPE phage library, and the amino acids sequences of C-CPE mutant were analyzed.
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of a CL binder based on the structural information. In the present study, we developed a novel screening system for CL binders by using a BV system and a C-CPE phage display library, and we used this system to identify novel CL4 binders.

In ligand screening, the preparation of a receptor for the ligand is very critical. Membrane proteins are especially difficult to prepare as recombinant protein with an intact structure. Functional membrane proteins such as cell-surface proteins are heterologously expressed on BV in their native forms [19–21]. Interactions between membrane proteins can be detected by using receptor-displaying and ligand-displaying BV [21]. In the present report, we found that CL4-BV interacts with a CL4 binder, C-CPE, but it does not interact with C-CPE303 that lacks the CL4-binding residues of C-CPE. The CL4-binding site of C-CPE corresponds to that of CPE; so, the second extracellular loop of CL appears to be the C-CPE-binding site [23,26]. These findings indicate that CL4 displayed on BV may have native form. We anticipate that CL-BV will be useful for the preparation of CL binders, such as peptides and antibodies.

To the best of our knowledge, the preparation of CL binder has been performed by only four groups. Offner et al. prepared polyclonal antibodies against extracellular domains of CL3 and CL4 [27], Ling et al. screened peptide types of CL4 binder by using a 12-mer peptide phage display library and CL4-expressing cells [28], Suzuki et al. generated a monoclonal antibody against the second extracellular loop of CL4 from mice immunized with a human pancreatic cancer cell line [29] and Romani et al. screened scFv against CL3 by using a human antibody phage display library [30]. However, the CL modulators have never been developed; thus, C-CPE is the only known CL4 modulator [12]. In the present study, we prepared a C-CPE phage library containing C-CPE mutants in which each of the 6 functional amino acids was randomly replaced with an amino acid, and we isolated CL4 binders by using CL4-BV as a screening ligand. Interestingly, all of
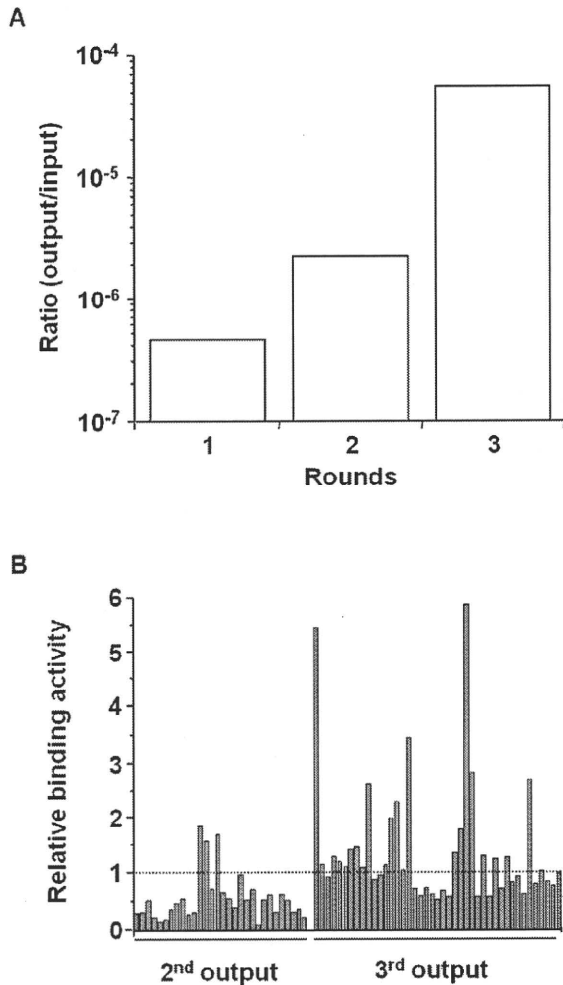
−600−

A

B

Table 2. CL4-binding phages.

	304	305	307	309	313	318
C-CPE	**S**	**S**	**S**	**N**	**S**	**K**
Clone 1	R	V	S	A	R	R
2	R	S	V	A	R	K
3	G	D	G	R	T	R
4	S	A	P	R	S	A
5	R	S	L	K	S	K

The sequences of C-CPE mutant in the CL4-binding phages were analyzed.
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or ZO-2 is developed, then useful and effective CL modulators can be identified. In this point, the BV system has extremely superior features. G protein and G protein-coupled receptors have been
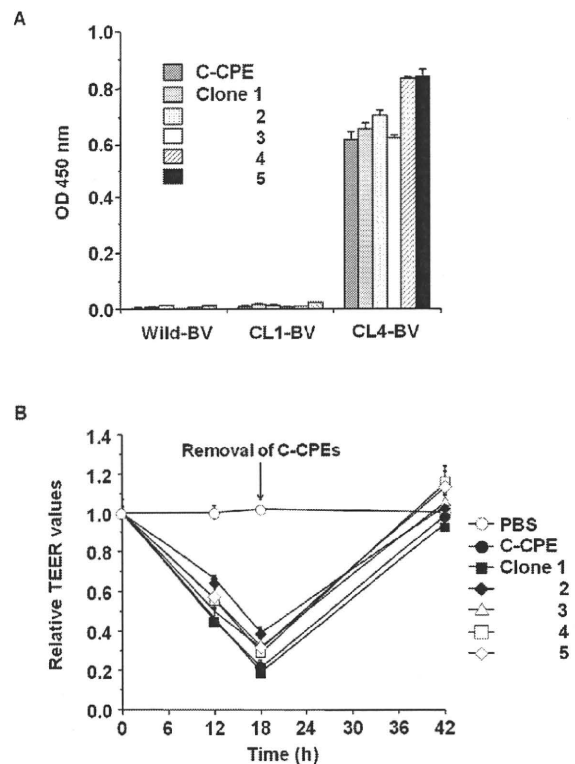
A

B

Figure 3. Screening of a novel CL4 binder. A) Enrichment of phages with affinity to CL4-BV. CL4-BVs coated on immunotubes were incubated with the C-CPE-derivative phage library at 1.6×10^{12} CFU titer (1st input phage). The phages bound to CL4-BV were recovered (1st output phage). The CL4-BV-binding phages were subjected to two additional cycles of the incubation and wash step, resulting in 2nd, 3rd output phage. The ratio of output phage to input phage titers was calculated. B) Monoclonal analysis of C-CPE-derivative phage. CL4-BV-bound phage clones were isolated from the 2nd and 3rd output phages, and the interaction of the monoclonal phage with CL4-BV was examined by ELISA with anti-M13 antibody as described in Materials and methods. Data are expressed as relative binding to that of C-CPE-phage indicated by the most right column.
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the CL4 binders modulated TJ barriers. We are investigating why the substitution of the amino acids with the other amino acids modulated CL4. These findings indicate that a BV screening system with a C-CPE library may be a powerful method to develop CL modulators.

The CL family forms various types of TJ barriers through combinations of its more than 20 members in homophilic/heterophilic CL strands [31,32]. Intercellular proteins ZO-1 and ZO-2 determine the localization of CL strands [33]. If a screening system to reconstitute heterogeneous CL strands with ZO-1 and/

Figure 4. Isolation of a novel CL4 modulator. A) Interaction of the C-CPE derivatives with CL4. C-CPE derivatives were prepared as his-tagged recombinant proteins. The C-CPE derivatives (0.02 μg) were added to CL-BV-coated immunoplates, followed by detection of the C-CPE derivatives bound to CL-BV. Data are means ± SD (n=4). B) Modulation of tight junction-barriers. Caco-2 cells were cultured on Transwell^{TM} chambers. When TEER values reach a plateau, the cells were treated with C-CPE or C-CPE derivatives at the indicated concentrations. After 18 h of exposure to the C-CPEs, the cells were washed with medium to remove C-CPEs, and then the cells were cultured for an additional 24 h. Changes in TEER values were monitored during the C-CPEs treatment. Relative TEER values were calculated as the ratio of TEER values at 0 h. Data are representative of two independent experiments. The data are means ± SD (n=4).
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functionally reconstituted in BV [20,34], and functional γ-secretase complexes have also been reconstituted on BV [35]. In the near future, the reconstituted CL system on BV will be developed and used for the screening of CL binders and modulators, hopefully leading to breakthroughs in pharmaceutical therapies that target CLs.

Materials and Methods

Recombinant BV construction and Sf9 cell culture

Recombinant BV was prepared by using the Bac-to-Bac expression system, according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD). Briefly, mouse CL1 and CL4 cDNA (kind gifts from Dr. M Furuse, Kobe University, Japan) were inserted into pFastBac1, and the resulting plasmids were transduced into DH10Bac E. Coli cells. Recombinant bacmid DNA was extracted from the cells. Sf9 cells were transduced with the bacmid coding CL, and the recombinant BV was recovered by centrifugation of the conditioned medium [36].

Preparation of the BV fractions

Sf9 cells (2×10^6 cells) were infected with recombinant BV at a multiplicity of infection of 5. Seventy-two hours after infection, the BV fraction was recovered from the culture supernatant of infected Sf9 cells by centrifugation. The pellets of the BV fraction were resuspended in Tris-buffered saline (TBS) containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and then stored at 4°C until use. The expression of CL1 and CL4 in the BV was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with anti-CL antibodies (Zymed Laboratory, South San Francisco, CA).

Preparation of mutant C-CPE library

C-CPE fragments in which the functional amino acids (S304, S305, S307, N309, S313 and K318) [24] were randomly mutated were prepared by polymerase chain reaction (PCR) with pET-H$_{10}$PER as a template, a forward primer (5′-catgccatggccgatatagaaaaagaaatccttgatt-tagctgctg-3′, Nco I site is underlined) and a reverse primer (5′-ttttccttttgcggccgcaaasnnttgaaataatatsnnataagggtasnnttccsnnatasnnsnnat-tagcttt-3′, Not I site is underlined, and the randomly mutated amino acids are in italics). The PCR fragments were inserted into a pY03 phagemid at the NcoI/NotI sites [22]. The resultant phagemid containing the C-CPE mutant library was transduced into E. coli TG1 cells, and then the cells were stored at −80°C.

Preparation of phage

TG1 cells containing phagemid coding a scFv, C-CPE, C-CPE mutant or C-CPE mutant library were culture in 2YT medium containing 2% glucose and ampicillin. When the cells grew to a growing phage, M13K07 helper phages (Invitrogen) were added, and the medium was changed into 2YT medium containing ampicillin and kanamycin. After an additional 6 h of culture, the phages in the conditioned medium were precipitated with polyethylene glycol. The phages were suspended in phosphate-buffered saline (PBS) and stored at 4°C until use.

ELISA

Wild-BVs or CL-BVs (0.5 μg/well) were adsorbed onto an immunoplate (Greiner Bio-One, Frickenhausen, Germany). The wells were washed with PBS and blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). C-CPEs or phages were incubated in the immunoplate, and the BV-bound C-CPEs or phages were detected by using anti-his-tag

antibody (Novagen, Darmstadt, Germany) or anti-M13 antibody (Amersham-Pharmacia Biotech, Uppsala, Sweden), respectively, horseradish peroxidase-labelled secondary antibody and TMB peroxidase substrate (Nacalai Tesque, Kyoto, Japan). The immunoreactive C-CPEs or phages were quantified by the measurement of absorbance at 450 nm. In the screening of phages, the data were normalized by the amounts of phages, which were quantified by ELISA for the FLAG-tag contained in the coat protein.

Selection of phage by using BV

A total of 0.5 μg of BV was adsorbed onto an immunotube (Nunc, Roskilde, Denmark). The tube was washed with PBS and blocked with TBS containing 4.0% BlockAce. The BV-coated tubes were incubated with mixture of phages, and then the tubes were washed 15 times with PBS and 15 times with PBS containing 0.05% Tween 20. The phages bound to the tube were eluted with 100 mM HCl. TG1 cells were infected with the eluted phages, and phages were prepared as described above. The resulting phages were subjected to repeated selection by using the BV-coated immunotubes.

Identification of a phage clone

To identify an isolated phage clone, we performed PCR or sequencing analysis. We amplified the inserted fragment into the phagemid by PCR using forward primer 5′-caggaaacagctatgac-3′ and reverse primer 5′-gtaaatgaattttctgtatgagg-3′. The resultant PCR products were subjected to agarose gel electrophoresis followed by staining with ethidium bromide. We performed a sequence analysis with primer 5′-gtaaatgaattttctgtatgagg-3′.

Measurement of phage titer

To quantify the concentration of phages, we measured the titer (colony formation unit (CFU)/ml) of the phage solution. Briefly, the phage solution was diluted to 10^{-5}–10^{-10} with PBS. The diluted solution was seeded onto Petrifilm™ (Tech-Jam, Osaka, Japan). After 24 h of incubation, the colonies were counted, and the titer was calculated.

Purification of C-CPE mutants

C-CPE and C-CPE303, in which the CL-4 binding region of C-CPE was deleted, were prepared as described previously [13]. To prepare plasmid containing C-CPE mutants, the C-CPE mutant fragment was PCR-amplified by using phagemids coding C-CPE mutants as a template. The resulting PCR fragment was inserted into pET16b, and the sequence was confirmed. The plasmids were transduced into E. coli strain BL21 (DE3), and production of mutant C-CPEs was induced by the addition of isopropyl-D-thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM MgCl$_2$, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol) that was supplemented with 8 M urea when necessary. The lysates were applied to HiTrap™ Chelating HP (GE Healthcare, Buckinghamshire, UK), and mutant C-CPEs were eluted with buffer A containing 100–400 mM imidazole. The buffer was exchanged with PBS by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80°C until use. Purification of the mutant C-CPEs was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-his-tag antibody (Novagen). Protein was quantified by using a BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, Rockford, IL).

−602−

TEER assay

Caco-2 cells were seeded in Transwell™ chambers (Corning, NY) at a subconfluent density. The TEER of the Caco-2 monolayer cell sheets on the chamber was monitored by using a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). When TEER values reached a plateau, indicating that TJs were well-developed in the cell sheets, the Caco-2 monolayers were treated with C-CPE or C-CPE mutants on the basal side of the chamber. Changes in TEER values were monitored. The TEER values were normalized by the area of the Caco-2 monolayer, and the TEER value of a blank Transwell™ chamber (background) was subtracted.

Author Contributions

Conceived and designed the experiments: MK TS TH KY. Performed the experiments: HK AT MK YS TY TS. Analyzed the data: HK AT MK KY. Contributed reagents/materials/analysis tools: HK AK TS TH. Wrote the manuscript: HK MK TY.

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