

Phage display selection on whole cells yields a small peptide specific for HCV receptor human CD81

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ABSTRACT

The human CD81 (hCD81), the most recently proposed receptor of hepatitis C virus (HCV), can specifically bind to HCV envelope glycoprotein 2 (E2). In this study, hCD81-expressing murine NIH/3T3 cells were used to select hCD81-binding peptides from a phage displayed nonapeptide library (PVIII9aaCys). Eighteen of the 75 clones selected from the library showed specific binding to the hCD81-expressing NIH/3T3 cells by enzyme linked immunosorbent assay (ELISA) and competitive inhibition test. Twelve out of the 18 clones shared the amino acid motif SPQYWGTGPA. Sequence comparison of the motif showed no amino acid homology with the native HCV E2. The motif-containing phages could competitively inhibit the ability of HCV E2 binding to native hCD81-expressing MOLT-4 cells, and induce HCV E2 specific immune response *in vivo*. These results suggest that the selected motif SPQYWGTGPA should be a mimotope of HCV E2 to bind to hCD81 molecules. Our findings cast new light on developing HCV receptor antagonists.

Key words: viral receptor, hepatitis C virus, cell-based selection, hCD81-binding peptide, phage display.

INTRODUCTION

Hepatitis C virus (HCV), a most common causative agent for post-transfusional hepatitis, is a positive-stranded RNA virus, classified as family *Flavivirida*[1]. Although the virus was identified more than a decade ago, some events in cell entry, replication and morphogenesis have not been well understood. It has been demonstrated that HCV envelope glycoprotein 2 (E2) could bind to host cells by interacting with the CD81 molecule [2]. CD81 is a membrane protein of 26 000 KD, and with four transmembrane (TM) and two extracellular (EC) domains, it belongs to the tetraspanin superfamily [3]. The larger extracellular loop (LEL) of CD81 molecule contains four conservative cysteine residues forming two disulfide bounds that maintain natural configuration of the protein. CD81 is expressed on most human cells and is demonstrated to play a role in cell adhesion, pro-

liferation, activation and differentiation[4-6]. Although the mechanism involving in the cell entry of HCV was not known clearly, human CD81 (hCD81) was considered as a putative HCV receptor[7-10]. Because no effective chemical reagents are available for the prevention of HCV infection, a phage displayed peptide library was used in this study to identify small peptides which are able to selectively block the interaction between hCD81 and HCV E2.

The phage display technique has some advantages in the study of protein-protein interaction[11-13]. And lots of antagonists were identified from phage displayed peptide libraries, such as urokinase receptor antagonists and the human type I interleukin (IL)-1 receptor antagonist [14-17]. However, there are some difficulties for membrane receptors to be purified and maintain the natural conformation. It is better to use whole native cells or target receptor gene transfected cells to select the ligands[18, 19]. Whole cells usually maintain the native conformation of receptor with normal posttranslational modification, so the ligands of receptor can be selected even without

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the information about the receptor.

In this study, hCD81-expressing murine NIH/3T3 cells were used to select hCD81-binding peptides from a phage peptide library. A small peptide specific for hCD81 was identified which is potential for development of HCV receptor antagonists.

MATERIALS AND METHODS

Cell lines

The murine fibroblast cell line NIH/3T3 (ATCC, Rockville, MD) were cotransfected with the eukaryotic expression vector pCDM8-hCD81 containing a full-length hCD81 cDNA (kindly provided by Dr. Levy S, Stanford University) and pSV2neo helper plasmid (ClonTech, Palo Alto, CA, USA) to obtain hCD81-expressing cells (NIH/3T3-hCD81)[20]. NIH/3T3-hCD81 and NIH/3T3 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 mg/ml of streptomycin. Human acute lymphoblastic leukemia cell line MOLT-4 and human hepatocyte line HL-7702, expressing native hCD81 molecules on cell membrane and hCD81-deficient cell line, human histiocytic lymphoma cells U937, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) FBS, 100 U/ml of penicillin and 100 mg/ml of streptomycin. Medium and supplements, with the FBS, were all from GIBCO-BRL, Gaithersburg, MD, USA.

Phage peptide library and bacterial strain

Phage peptide library (PVIII9aaCys) was composed of random circular nonamers, which were fused to the N terminus of the major coat protein PVIII displayed on filamentous phage f1[21]. Host bacterial strain XL1-blue was from Stratagene Company, Cambridge, England.

Selection of phage peptide library with NIH/3T3 hCD81 cells

Selection procedure was according to Pelsers and Souriau with some modifications[22, 23]. Briefly, phages of approximate 4×10^{10} phage transducing unit (TU) were preincubated for 1 h with blocking-buffer (5 mg/ml bovine serum albumin (BSA), 0.1 mol/L NaHCO_3) and then with 1×10^6 of wild type NIH/3T3 cells. After centrifugation at 10,000 rpm for 5 min at 4°C, the supernatant was transferred to a 35 mm cell culture plate (Nunc, Roskilde, Denmark), on which 3.5×10^5 NIH/3T3-hCD81 cells were grown, and incubated for 2 h. Unbound phage particles were removed by washing with 0.1% TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). Cell-bound phages were eluted with 100 mM Glycine-HCl (pH 2.2) for 10 min, and neutralized with 1 M Tris-HCl (pH 9.0). The eluted phages were replicated by infecting *Escherichia coli* XL1-blue cells, rescuing with M13KO7 helper phages (Pharmacia Biotech, Uppsala, Sweden). The amplified phage particles were purified using polyethylene-glycol (PEG)[24], and then used for the subsequent round of selection with NIH/3T3-hCD81 cells. Four rounds of selection were performed as above.

Whole-cell ELISA

The exponentially growing NIH/3T3, NIH/3T3-hCD81 or MOLT-4 cells were fixed on 96-well microtitration plates (Nunc, Roskilde, Denmark) (about 3×10^5 cells/well) with 1 mg/ml of polylysine. Amplified phage clones were randomly picked after the fourth round of selection with the preincubation for 1 h with 3% BSA. About 2×10^{11} TU of phages were added to each well, and then incubated with the cells for 2 h at room temperature. The wells were washed extensively with 0.1% TBST and the amount of bound phages was detected with horseradish peroxidase (HRP)-conjugated anti-M13 phage antibody (Pharmacia Biotech, Uppsala, Sweden). The development was performed by the addition of 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma, St. Louis, MO, USA), and read at 450 nm in an ELISA Reader (Bio Rad). Helper phage M13KO7 and original phage peptide library PVIII9aaCys were used as negative controls.

Competitive ELISA

MOLT-4 cells were fixed about 3×10^5 cells per well on 96-well microtitration plates using polylysine. Anti-hCD81 monoclonal antibody (Pharmacia) and positive phage particles (5×10^{12} TU/ml) were mixed and then added to each well. After 1 h of incubation, the wells were washed with 0.1% TBST, and incubated at 37°C for 1 h in the presence of HRP-conjugated anti-M13 phage antibody (1:5000 diluted). Finally, the wells were developed with substrate TMB, and read at 450 nm.

Sequence analyses

DNA phagemids were prepared from identified phage clones by standard method[25] and sequenced with ABI Prism kit (Perkin Elmer Applied Biosystems, USA) automatically. The primer used for sequencing is 5'-CCCACGCATAACCGATA-3'. Corresponding amino acid sequences were deduced from DNA sequences, and a multiple sequence alignment was done using BLAST software package provided by <http://ncbi.nlm.nih.gov/BLAST/> to determine the groups of related peptides. Homologous analysis was performed to find an optimal alignment between the selected motifs and the primary sequence of HCV E2.

Specific analyses of positive phages binding to hCD81 protein

Competitive inhibition test

Native hCD81-expressing MOLT-4 cells were fixed on 96-well microtitration plates about 3×10^5 cells per well and blocked for 1 h with 3% BSA. HCV E2 antigen (kindly provided by Dr. Donnelly III JJ, Chiron Company, USA) and positive phages in serial dilutions were added to each well and incubated for 1 h at room temperature. After washing with 0.1% TBST, the cell-bound phages were detected with HRP-conjugated anti-M13 phage antibody (Pharmacia Biotech, Uppsala, Sweden). And the cell-bound HCV E2 was detected with mouse anti-HCV E2 mAb (kindly provided by Dr. Donnelly III JJ) and HRP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, USA).

Immunohistochemistry

Exponentially growing MOLT-4, HL-7702 or U937 cells (about 3.5×10^5 cells per well) were cultured respectively with positive phage particles (about 1×10^{11} TU) for 2 h at room temperature. The cells were washed extensively with phosphate-buffered saline (PBS), swung onto the glass slides, and then fixed with acetone. The glass slides were incubated with HRP-conjugated anti-M13 phage antibody at 37°C for 1 h. After washed with PBS, the slides were developed with diaminobenzidine (DAB), counterstained lightly with hematine crystal, dehydrated through a graded series of ethanol to xylene, and coverslipped with permount.

Immunization of mice and ELISA

The purified positive phage particles were resuspended with 0.9% NaCl at a concentration of 1×10^{13} TU/ml. Four-to-six-week old female C57BL/6 mice (Experimental Animal Center, SMMU, China) were immunized by i. p. injection with 200 µl of positive phage emulsion (1:1 with complete Freund's adjuvant). Same dosages with incomplete Freund's adjuvant were used for the booster at weeks 4 and 7. The animals were bled for titer detection on the 10th day after second and third injections.

Microtitration plates were coated with 0.1 µg/well of HCV E2 and blocked with 3% BSA for 2 h at 4°C. 150 µl mouse serum of different dilutions was added to each well, and incubated for 1 h at 37°C. After washed with PBS, the cells were incubated with HRP-conjugated goat anti-mouse IgG (Sigma) for 40 min at 37°C. The development was performed by addition of substrate TMB, and read at 450 nm. For competition assay, 150 µl of positive serum (diluted 1:100) from immunized mice were mixed with a graded series of HCV E2 antigen and added to HCV E2 coated microtitration plates. Normal mouse serum and equal volume of PBS were used as controls.

RESULTS

Specific enrichment of positive phages

In order to enrich hCD81-binding phages from the phage peptide library, four rounds of selection with NIH/3T3-hCD81 cells were performed. The enrichment was determined by the use of the output/input ratio of phages after each round of selection. The ratio increased about 8-fold (from 2.3×10^{-6} to 1.9×10^{-5}) after the second round

Tab 1. Enrichment of phages for each round of selection from phage displayed nonapeptide library

Rounds	Selected phages (TU*) (input)	Eluted phages (TU) (output)	Ratio (out/input)
1	4.8×10^{10}	1.1×10^5	2.3×10^{-6}
2	4.2×10^{10}	8.0×10^5	1.9×10^{-10}
3	3.3×10^{10}	2.2×10^7	6.7×10^{-4}
4	4.0×10^{10}	3.7×10^8	9.3×10^{-3}

* TU: transducing unit

of selection. After the third and the fourth rounds of selection, the output/input ratio of phages increased about 290-fold and 4,000 fold, respectively (Tab 1), which indicates an obvious enrichment for the specific binding of phages to hCD81-expressing cells.

Identification of the positive phages

75 clones were picked out from the sample after fourth round of selection, and the specificity was examined by whole-cell ELISA. Thirty of the 75 clones (40%) showed the binding ability to NIH/3T3-hCD81 cells and native hCD81-expressing MOLT-4 cells (data not shown). In contrast, the original phage library and helper M13KO7 phages cannot bind to the above 2 types of cells. Competitive inhibition test further demonstrated that 18 out of the 30 positive clones could specifically inhibit the binding of anti-hCD81 MAb to MOLT-4 cells. Mean inhibition ratio of the 18 clones was 70% (Fig 1).

Analyses of exogenous sequences of positive phage clones

The exogenous DNA sequences of 18 positive phage clones were determined by DNA sequencing. The deduced

Tab 2. Exogenous amino acid sequences (deduced from DNA sequences) of the nonapeptides in PVIII coat proteins of positive phage clones selected from PVIII9aaCys peptide library

Phage clones	Amino acid sequences
C4	C SPQYWTGPA C
C9	C SPQYWTGPA C
C14	C SPQYWTGPA C
C20	C SPQYWTGPA C
C25	C SPQYWTGPA C
C28	C SPQYWTGPA C
C38	C SPQYWTGPA C
C53	C SPQYWTGPA C
C58	C SPQYWTGPA C
C60	C SPQYWTGPA C
C65	C SPQYWTGPA C
C73	C SPQYWTGPAC
C7	C VQFPTSEKM C
C35	C SDPRKMCY C
C41	C IWENAGRMV C
C46	C HAGTFLQVA C
C54	C LVAQINLEM C
C64	C ERHTKFPSV C

Each end of the exogenous nonapeptides is linked with one cysteine residue (C). Single letter abbreviations of amino acids: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Glu; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

peptide sequences from DNA sequences were shown in Tab 2. Of the 18 clones, 12 shared the motif SPQY-WTGPA. The motif did not show any homology to HCV E2, which implied that the motif should be a mimic sequence of HCV E2 able to bind to hCD81 molecules on cell membrane.

The motif-containing phages specific to hCD81 on cell membrane

The interaction of the motif-containing phages and several human cell samples was detected by immunohistochemistry staining. Positive phages showed binding to MOLT-4 cells (Fig 2A) and HL-7702 cells (Fig 2B), and dark brown positive staining was seen with phage antibodies on the surfaces of these cells. In contrast, no positive staining was observed on the hCD81-deficient U937 cells (Fig 2C). Primary phage peptide library without selection was used as negative control, while, no positive staining was observed on the surfaces of MOLT-4 cells (Fig 2D), HL-7702 cells (Fig 2E) and U937 cells (Fig 2F).

The ability of the motif-containing phages to block the binding of the hCD81-expressing cells with HCV E2 was

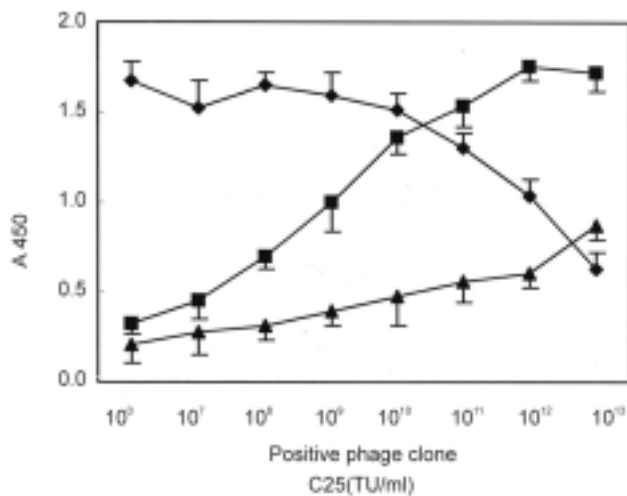


Fig 3. Competitive binding inhibition between positive phages and MOLT-4 cells by HCV E2 protein. MOLT-4 cells were fixed on 96-well microtitration plates using polylysine. Inhibition of HCV E2 binding to MOLT-4 cells was observed by increasing titers of positive phage clone C25. The cell-bound phages were detected by ELISA with horseradish peroxidase (HRP)-conjugated anti-M13 in the presence (▲) or absence (■) of HCV E2. The cell-bound HCV E2 were detected by anti-HCV E2 and HRP-conjugated goat anti-mouse IgG (◆). The data represent the mean values of triplicate measurements from a single experiment; error bars represent the standard error of triplicate samples. Comparable data are obtained in three independent experiments.

determined by competitive ELISA. The phage clone C25, with the highest (81%) effect to block the binding of anti-hCD81 MAb to MOLT-4 cells, was chosen for the test. It was found that the binding ability of the clone C25 with MOLT-4 cells increased with the increasing amount of the phages. However, the binding of the phages with MOLT-4 cells decreased significantly in the presence of HCV E2 (2 mg/ml). Similarly, the binding of HCV E2 to MOLT-4 cells was inhibited by the motif-containing phages in a dose-dependent manner too (Fig 3).

Immunogenic mimicry of HCV E2 epitope

The motif-containing phages were used for the immunization of C57BL/6 mice. Ten days after the third immunization, the mouse sera were collected and analyzed by titer detection. The sera could react with HCV E2. Furthermore, the reaction of HCV E2 with the sera decreased significantly in the presence of HCV E2 antigen (Fig 4).

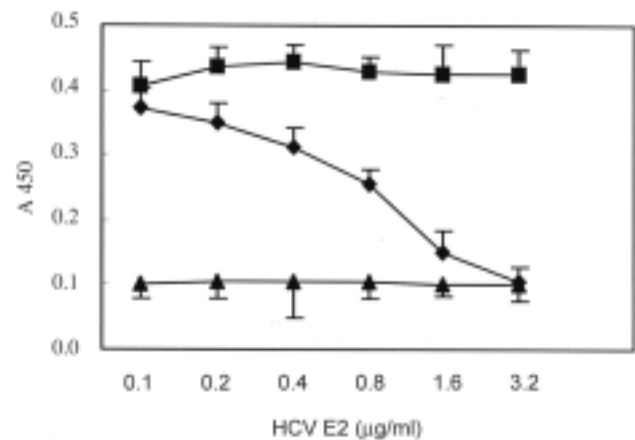


Fig 4. Competitive ELISA reactivity of HCV E2 with the sera from the mice immunized with phage clone C25 in the presence of HCV E2. HCV E2 antigen was coated onto microtitration plates. The binding of HCV E2 to the sera (diluted 1/100) from the mice immunized with positive phages was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG by the addition of different concentrations of HCV E2 antigen (◆) equal volume of solvent PBS (■). Normal mouse serum (diluted 1/100) was used as a control (▲). The data represent the mean values of triplicate measurements from a single experiment; error bars represent the standard error of triplicate samples. Comparable data are obtained in three independent experiments.

DISCUSSION

It has been proved that the phenylalanine (Phe) at amino acid (aa) 186 of hCD81 large extracellular loop plays a

key role in recognition for CD81 binding to HCV E2. Binding site of HCV E2 for hCD81 is a conformational structure naturally involving aa 480 to 493 and 544 to 551 within the E2 protein[3, 7, 26]. Thus it is difficult for a linear peptide library to mimic the discontinuous epitopes of HCV E2 protein. In this work, a conformational constrained peptide library was used, in which each end of the exogenous random nonapeptides is linked with one cysteine residue, so circular disulfide-bonded constrained conformation can be formed in the peptide library. It has been reported that the phage displayed constrained library has high affinity with receptor and is easy to be selected for functional peptides or structural mimetics of proteins[16, 27, 28]. Wrighton and colleagues have obtained the ligands of erythropoietin (EPO) receptor from this kind of phage random circular octapeptide library[29]. However, some cell membrane receptors such as CD81 are rarely used possibly because it is difficult for them to maintain the natural conformation or topostucture. Therefore, we established an hCD81-expressing NIH/3T3 cell line for the selection of hCD81-binding peptides from the phage peptide library. The major problem existed in cell-based selection is that phage displayed peptides can bind to the modified cells nonspecifically. In order to overcome the problem, multi-rounds of selection were carried out in our experiment according to Smith's method[30]. In addition, before each round of selection, wild type hCD81-deficient NIH/3T3 cells were used for preincubation with the library in order to remove the non-specific phages. Specific phages for binding to hCD81-expressing cells were obtained from the phage peptide library and further enriched round by round.

The phage clones were further identified after the fourth round of selection by whole-cell ELISA and competitive inhibition test. The binding activity between 18 positive clones and hCD81-expressing MOLT-4 cells could be inhibited by the anti-hCD81 MAb. It has been proved that the MAb used in this study can block the site of HCV E2 binding to hCD81[2], thus indicating these 18 clones should be of the same determinant as HCV E2. Although the DNA sequences of these 18 clones were not identical, 12 out of the phage clones displayed the amino acid motif SPQYWTGPA. The motif did not show any homology to the primary sequence of native HCV E2 as we had expected. However, the immunohistochemistry staining showed that the motif-containing phages could bind to MOLT-4 and HL-7702 cells,

both of which express native hCD81, but do not bind to hCD81-deficient U937 cells. Furthermore, the motif-containing phages could inhibit the ability of HCV E2 binding to MOLT-4 cells in a competitive manner. These results demonstrate that the selected motif SPQYWTGPA should be a mimotope of HCV E2 to bind to hCD81 molecules.

If the motif displayed on the selected phages mimics HCV E2 epitope, it should be immunogenic mimics of the natural antigen. To verify this inference, the purified motif-containing phage particles were used as antigen to immunize C57BL/6 mice. Compared with the control, the immunized mice showed an obvious humoral immune response against HCV E2 antigen. The specificity of the response was further identified by a competition assay, and the results showed that the reaction of the phages with the mouse serum was significantly inhibited in the presence of HCV E2. These results suggest that the motif SPQYWTGPA might mimic the structural epitope of HCV E2 and provide the potential for further development of HCV receptor antagonists.

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