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Original Article

Identification of a Target Antigen Recognized by a Mouse Monoclonal Antibody to the Bile Canalicular Surface of Rat Hepatocytes with a Random Phage Display Library

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We developed a monoclonal antibody (MoAb) (clone 5E8) against an antigen on the bile canalicular membrane of rat hepatocyte. By immunoblotting, MoAb 5E8 detected a band of 110 kD. In this study, we used the phage display technique to identify the target antigen recognized by MoAb 5E8. We screened a random phage display library expressing 12-mer peptide sequences and identified a peptide sequence, FHFNPYTGHPLT, as an epitope. We compared this peptide sequence with those of dipeptidyl peptidase IV (DPP IV, E.C.3.4.14.5) and Cell-CAM105, which proteins were located by a database search based on the information of tissue localization and approximate molecular weight of the MoAb 5E8 antigen, and sequence similarity with a region in DPP IV (amino acids 225-233) but not with Cell-CAM105 was found. In addition, we immunohistochemically stained various tissues (liver, small intestine, and kidney) of Japanese Fischer 344 rats, known to be deficient for DPP IV, with MoAb 5E8 and showed that the expression of MoAb 5E8 antigen was negligible or weak. In contrast, tissues sampled from the same organs of Sprague-Dawley rats, known to express DPP IV, were positively stained. These findings suggest that the antigen recognized by MoAb 5E8 is DDPIV and its major epitope is located in amino acids at positions 225-233.

Key words: random phage display library, dipeptidyl peptidase IV, monoclonal antibody, epitope, bile canalicular membrane

Hepatocytes are polarized epithelial cells with 3 morphologically and functionally distinct surface domains: the sinusoidal, the lateral, and the bile canalicular surface domains. We previously developed 3 kinds of mouse monoclonal antibodies that react with 3 different respective antigens on the surface of rat hepatocytes [1]. One of them, monoclonal antibody (MoAb) (clone 5E8), recognized an antigen present on the bile canalicular membrane but absent from the sinusoidal and lateral plasma membranes. Immunohistochemical staining of other organs of rats showed that the antigen detected by MoAb 5E8 was present on the apical surfaces of epithelial cells in the pancreas, small intestine, colon, and bile duct, and on the luminal portions of the proximal renal tubule cells, and, weakly, on the glomeruli. By immunoblot analysis, MoAb 5E8 detected a band with an approximate molecular weight of 110 kD.

Random peptide libraries constructed by fusion of

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random sequences to the amino-terminal region of pIII of filamentous bacteriophages [2] have been used in a number of applications including identification of epitopes recognized by monoclonal or polyclonal antibodies (reviewed in [3]). In this study, we adapted this technique to identify the target peptide recognized by MoAb 5E8. In addition, we performed a database search for possible target protein(s) of MoAb 5E8, based on the information of tissue localization and approximate molecular weight, and investigated sequence similarity between the peptide selected from phage display libraries and the possible target protein selected from the database.

Materials and Methods

Preparation of MoAb5E8. MoAb 5E8 (IgG1 subclass) to an antigen present on the bile canalicular membrane of rat hepatocytes was prepared as previously described [1] and purified from ascitic fluids of pristane (Aldrich, Milwaukee, WI, USA)-treated BALB/C mice bearing 5E8 hybridoma using protein G-agarose gel affinity chromatography (MAb Trap Kit; Amersham Pharmacia Biotech Ltd., Buckinghamshire, England).

Phage Display Library. As a random phage library, we used the Ph.D.-12 Peptide Library Kit (New England BioLabs, Inc., Beverly, MA, USA). This kit is based on a combinatorial library of random peptide 12-mers fused to a minor coat protein (pIII) of the filamentous coliphage, M13. The Ph.D.-12 peptide library consists of 1.9×10^9 independent clones.

Screening of MoAb 5E8 binding phage in a random peptide library (biopanning). Phage virions (4×10^{10}) and MoAb 5E8 (300 ng) in 200 μl of TBS-T buffer (50 mM Tris-HCl, Ph 7.5, 150 mM NaCl (TBS), and 0.1% Tween 20) were incubated at room temperature for 20 min, then the mixture was reacted with 50 μl of Protein A-Sepharose beads (Amersham Pharmacia Biotech Ltd.)(50% suspension in TBS) at room temperature for 15 min. After washing with TBS-T, bound phage was eluted with 1 ml of 0.2 M glycine-HCl, pH 2.2, containing 1 mg/ml bovine serum albumin (BSA). Phage elution was immediately neutralized with 150 μl of 1 M Tris-HCl, pH 9.1, then amplified in 20 ml of *E. coli* host strain ER2537. After centrifugation, 20% polyethylene glycol-8000, 2.5 M NaCl was added to the supernatant, and amplified phages were collected. Using the amplified phage, the second round of biopanning was carried out using Protein G-Sepharose beads instead of Protein A-Sepharose beads. We carried out 4 rounds of biopanning in total, using Protein A and G beads alternatively. Fortuitous selection of peptide sequences that bound protein A or protein G could be avoided by these alternating rounds of Protein A- and G-Sepharose. Selected clones were subjected to DNA sequence analysis with an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the dye terminator dideoxy method (ABI PRISM Dye Terminator Cycle Sequencing FS Ready Reaction Kit, Applied Biosystems) according to the manufacturer's protocol.

Phage ELISA. Binding of selected phage peptides to MoAb 5E8 was further analyzed by enzyme-linked immunosorbent assay (ELISA) to confirm the affinity and specificity. Microtiter plates (Maxisorp, Nunc, Denmark) were coated with MoAb 5E8. As control, anti-FLAG M2 MoAb (IgG1 subclass, EASTMAN KODAK COMPANY, New Haven, CT, USA), which binds to FLAG fusion proteins expressing the amino acids sequence DYKDDDDL, was used. Plates were blocked with 0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml BSA, 0.02% NaN_3, and subsequently incubated with selected phages (10^9/well). After washing, bound phages were detected with horseradish peroxidase (HRP)-conjugated anti-M 13 antibody (Amersham Pharmacia Biotech Ltd.) and 2,2'-azino-di-3-ethylbenzo-thiazoline-6-sulphonic acid as substrate. Optical densities at 415 nm were measured on an automated ELISA plate reader.

Search for candidate proteins recognized by MoAb 5E8 and homology search. Based on the information of tissue localization and approximate molecular weight of the antigen detected by MoAb 5E8, we searched a database of the National Center for Biotechnology Information for possible target protein(s) of MoAb 5E8. Then, the sequence similarity between the possible target proteins and selected clones was investigated by a computerized homology search using the homology search software "GENETYX-MAC" (Software Development Co., Ltd., Tokyo, Japan).

Immunohistochemistry. Adult male Japanese-strain Fischer 344 rats and Sprague-Dawley rats were obtained form Charles River Japan, Inc. (Kanagawa, Japan). Cryostat sections of various tissues of these rats were fixed with cold acetone and reacted with MoAb 5E8. After washing, the sections were incubated with HRP-

labeled Fab' fragments of rabbit anti-mouse immunoglobulins [4], then with diaminobenzidine solution containing hydrogen peroxide.

Results

Identification of Peptides Binding to MoAb 5E8 Using a 12-mers Phage Display Library. A total of 11 clones obtained after the fourth round of biopanning were randomly chosen and subjected to DNA sequence analysis. The deduced amino acid sequences of selected phages are shown in Table 1. Samples 2 (phage 2) and 7 (phage 7) were identical. These 11 clones were subjected to analysis by phage ELISA. Only these 2 clones (phages 2 and 7) were found to be reactive to MoAb 5E8 but not to control anti-FLAG M2 MoAb in the ELISA assay (Fig. 1). The amino acid sequence of these clones was FHFNPYTGHPLT.

Homology Search. Database search identified dipeptidyl peptidase IV (DPP IV, E.C.3.4.14.5) and Cell-CAM105 as proteins that were present on the bile canalicular cell surface of rat hepatocytes and had an approximate molecular weight of 110 kD. Sequence similarity between these 2 proteins and the selected clones was investigated. Although no clear consensus sequence was observed in Cell-CAM105, it was found that phages 2 and 7 showed sequence similarity with a region in DPP IV (amino acids 225–233; an alignment of 60% / 10 amino acids) (Fig. 2).

Immunohistochemistry. Japanese-strain Fischer 344 rats have been reported to be deficient for DPP IV [5–7], whereas Sprague-Dawley rats are known to express this enzyme. Thus, we examined the expression of the target antigen of MoAb 5E8 in Fischer 344 rats and Sprague-Dawley rats immunohistochemically. In immunohistochemical staining with MoAb 5E8, staining of tissues of Japanese Fischer 344 rats was negligible in the liver and the kidney, and very weak in the small intestine. In contrast, MoAb 5E8 reacted with the bile canalicular channels of hepatocytes, the brush borders of epithelial cells of the small intestine, the luminal portion of the proximal renal tubules, and the glomeruli of Sprague-Dawley rats (Fig. 3).

Discussion

In this study, we used the phage display technique to identify a target antigen recognized by MoAb 5E8 that we

Table 1 The deduced amino acid sequences of clones isolated from a phage display library

Clone	Sequence
phage 1	KVVPPWHSVLPG
phage 2	**FHFNPYTGHPLT**
phage 3	SPKLLTNWHLLI
phage 4	HNWGGSDFWSFG
phage 5	FDPNNYWTPMGR
phage 6	TDMQFPGFHAAL
phage 7	**FHFNPYTGHPLT**
phage 8	EYPDPPAWHLWF
phage 9	WSATWTLSDTWK
phage 10	YTTPPFHTGWIW
phage 11	YHAGRVNTMSWL

Boldface type, identical clones.

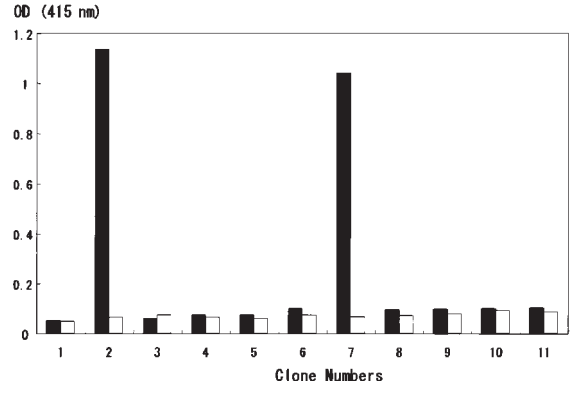

Fig. 1 Selection of MoAb 5E8 binding phage by an ELISA. Eleven clones randomly chosen after the fourth round of biopanning were subjected to analysis by an ELISA. ELISA plates were coated with either MoAb 5E8 (solid bars) or irrelevant control IgG1 anti-FLAG M2 MoAb (open bars) and reacted with clones. Two clones (phages 2 and 7) specifically bind to MoAb 5E8.

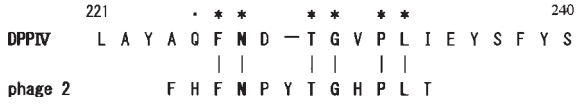

Fig. 2 Amino acid sequence alignment of phage 2 and the DPP IV molecule. Numbering indicates the amino acid position in DPP IV molecule. Asterisks and a period indicate identical and similar amino acids corresponding to the sequence of phage 2, respectively.

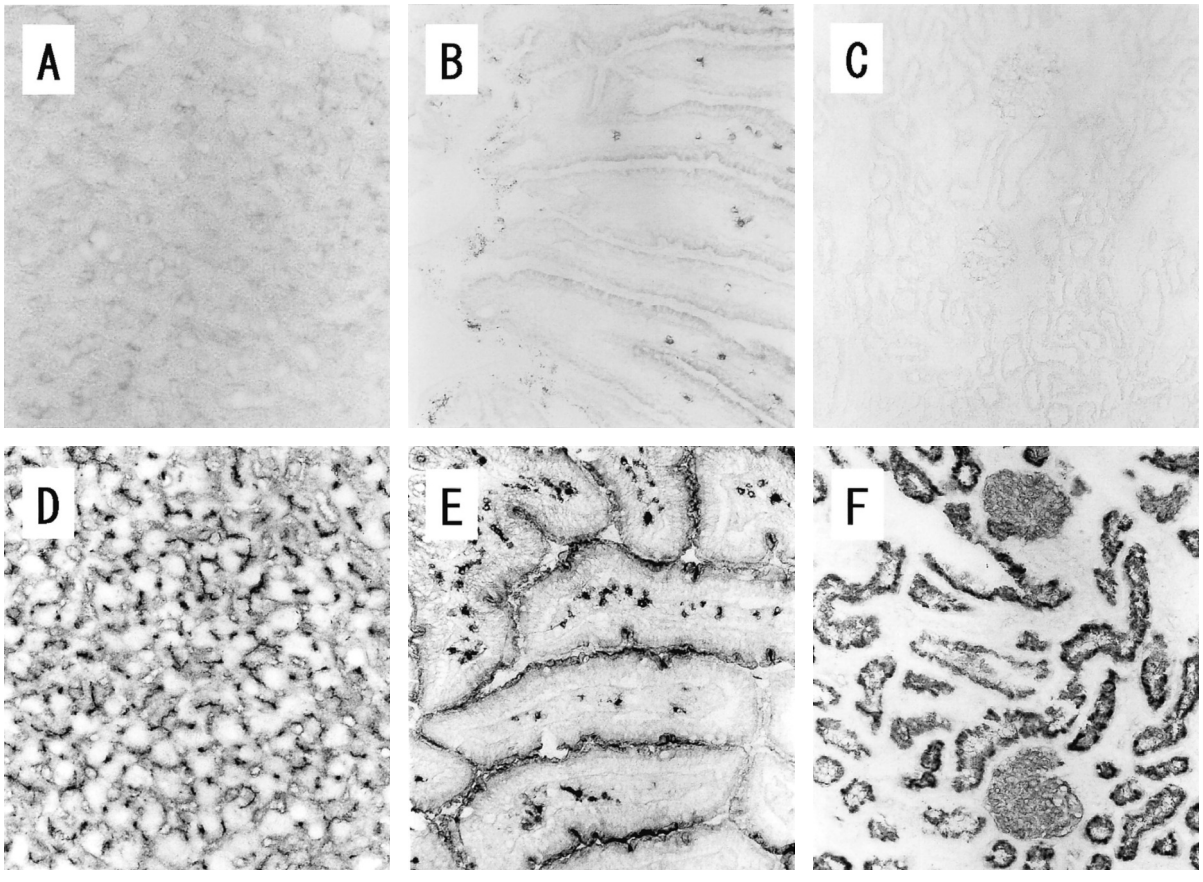

Fig. 3 Immunohistochemical staining of tissues of Japanese Fischer 344 rats deficient for DPP IV with MoAb 5E8. Cryostat sections of livers (**A**, **D**), small intestines (**B**, **E**), and kidneys (**C**, **F**) of adult male Fischer 344 rats (**A**, **B**, **C**) and Sprague-Dawley rats (**D**, **E**, **F**) were immunohistochemically stained with MoAb 5E8. Staining of tissues of Fischer 344 rats is negligible (**A**, **C**) or very weak (**B**). In contrast, the bile canalicular channels of hepatocytes (**D**), the brush borders of epithelial cells of the small intestine (**E**), the luminal portion of the proximal renal tubules, and the glomeruli (**F**) of Sprague-Dawley rats are positively stained with MoAb 5E8.

had previously generated as an antibody to the bile canalicular membrane of rat hepatocytes [1]. We screened a random phage display library expressing 12-mer peptide sequences and identified a peptide sequence, FHFNPYTGHPLT, as an epitope. This library contains 1.9×10^9 independent clones but represents a small sampling of the potential sequence space of 20^{12} $(= 4.1 \times 10^{15})$ 12-mer sequences. However, the library permits affinity selection of peptide ligand requiring tight binding. Additionally, the increased length of the displayed peptide allows folding into small structure elements such as short helices and isolation of sequences epitopes even if a monoclonal antibody recognizes its antigen in a conformation-dependent manner.

In our previous work, we defined the tissue localization and approximate molecular weight of the target antigen recognized by MoAb 5E8. This information enabled us to identify 2 proteins as possible antigens recognized by MoAb 5E8 based on a database search. One was Cell-CAM 105, a cell surface glycoprotein involved in intercellular adhesion of rat hepatocytes [8], which has been postulated to play a role in liver histogenesis [9]. The other was DPP IV, a serine-type protease that removes the amino-terminal dipeptide from peptide substrate provided that the penultimate amino acid residue is proline or alanine [10]. We compared the sequences of these 2 proteins with the peptide sequence FHFNPYTGHPLT, and found sequence similarity with a region in DPP IV (amino acids 225–233) but not with any region in Cell-CAM105. These findings suggest that the antigen of MoAb 5E8 is DPP IV, and that its major epitope is located among amino acids at positions 225–

233. We confirmed this conclusion immunohistochemically by staining tissues of rats with and without the expression of DPP IV, respectively, with MoAb 5E8.

DPP IV is widely distributed in a number of mammalian tissues [11–13] and is suggested to play an important role in various kinds of biological processes. DPP IV in the bile canalicular membrane is involved in the metabolism of circulating substance P [14, 15] and fibronectin-mediated interactions of hepatocytes with the extracellular matrix [11, 16]. Intestinal DPP IV plays a role in the hydrolysis of prolyl peptides and assimilation of proline-rich protein [17, 18]. DPP IV in the renal brush-border membrane hydrolyzes the filtered oligopeptides, — particularly the proline-rich, collagen-derived peptides — to generate dipeptides, and the resulting small peptides are then transported into the vesicles by the transport system [19, 20]. Thus, DPP IV plays an important role in the reclamation of peptide nitrogen from larger peptides. In this study, we demonstrate that MoAb 5E8 recognizes DPP IV. We hope that the antibody will be utilized for exploring further roles of DPP IV in biological processes as wells as the mechanisms by which the enzyme functions.

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