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Generation of a cholangiocyte-specific cDNA expression library for the identification of B and T cell autoantigens in murine biliary disease

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Abstract

Aim—Several mouse models of inflammatory cholangiopathies exist, including biliary atresia, primary biliary cirrhosis, autoimmune hepatitis, and primary sclerosing cholangitis. In an ongoing effort to identify the target antigens of both infiltrating autoreactive T cells and serum autoantibodies, we aimed to generate a cholangiocyte-derived cDNA library capable of expressing a wide variety of proteins.

Methods—mRNA was isolated from a normal mouse cholangiocyte cell line and reverse transcribed into cDNA. After initial cloning of the cDNA into a transfer vector (pDONR222), the entire library was shuttled into an *Escherichia coli* expression vector (pDEST160).

Results—The library contains 2.3×10^6 independent clones and expresses proteins up to 100 kD in molecular weight. Using a variety of techniques, including western blot analysis, mass spectrometry of individual clones, and direct DNA sequencing of plasmids, a number of both ubiquitously expressed and cholangiocyte-specific proteins (e.g. cytokeratin 19) have been identified within.

Conclusion—A comprehensive mouse cholangiocyte cDNA expression library has been generated and is available for use as a source of multiple cholangiocyte-specific antigens for immunological studies. The library can be used to screen for specificity of T cell lines or hybridomas. Furthermore, this library has potential uses in SEREX analysis of autoantibody reactivity. The cholangiocyte-specific cDNA library is a powerful tool for the identification of target antigens in murine inflammatory cholangiopathies and is available as a shared resource.

Keywords

autoimmune response; bile duct diseases; cDNA library; serological analysis of recombinant cDNA expression libraries

INTRODUCTION

Autoimmune diseases of the bile duct, such as primary sclerosing cholangitis (PSC),^{1–3} primary biliary cirrhosis (PBC),^{1,4,5} and autoimmune cholangitis⁵ are likely to be the result of autoantigen-specific reactivities of T and/or B lymphocytes. Evidence exists that biliary

atresia (BA) may also involve autoimmune-mediated bile duct injury.^{6,7} Recent investigations have demonstrated bile duct-targeted autoreactive T cells in BA mice^{8,9} and serum autoantibodies, including α -enolase autoantibodies.⁷ However, the exact cholangiocyte protein targets of the T cells and the many other serum autoantibodies has yet to be elucidated. The ability to determine what cholangiocyte proteins are targeted would greatly affect the ability of researchers to understand the progression of disease in BA as well as other inflammatory cholangiopathies.

A useful tool in identifying potential mouse cholangiocyte autoantigens would be an expression cDNA library representing multiple proteins present in cholangiocytes. These proteins could then be assayed for their reactivity to serum from autoantibody-producing mice by serological analysis of recombinant cDNA expression libraries (SEREX).¹⁰ SEREX has emerged as a powerful tool in identification of novel autoantigens in both cancer and autoimmune disease.^{11–13} In addition to tracking the humoral response, these proteins can also be used to identify T-cell antigens in autoimmune disease.¹⁴ Total murine liver cDNA libraries are currently commercially available, but as cholangiocytes are only 3% of the total mass of the liver,¹⁵ the overwhelming majority of antigens present in those libraries are not cholangiocyte specific. In order to ensure that cholangiocyte autoantigens would be adequately represented, we generated an expression cDNA library using a normal mouse cholangiocyte cell line as the source of mRNA.

METHODS

Isolation of cholangiocyte RNA and production of cDNA library

Mrna from an sv40 large T antigen-immortalized normal mouse cholangiocyte cell line (NMC, gift of Y. Ueno) was extracted with a Fast-Track 2.0 kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Purified mRNA was converted to cDNA following the protocol of the Clone-Miner cDNA library construction kit (Invitrogen). Following ligation of recombination adapters, cDNA was cloned into the Gateway vector pDONR222. The library was analyzed for average insert size by restriction enzyme digestion of individual clones with *BsrGI* (New England Biolabs). Titer of initial library was determined by plating dilutions of library on LB plates containing carbenicillin (50 μ g/mL) and performing colony counts.

Transfer of cDNA library to expression vector

Library was transferred via site-specific recombination to the *Escherichia coli* expression vector pET160-DEST following the protocol of the Champion pET Gateway Expression Kit (Invitrogen). Library was electroporated into E.coli EXPRESS BL21 cells (Lucigen, Middleton, WI, USA). Library in pET160 was amplified in semisolid culture as follows. Two liters of 2xLB, 0.3% w/v SeaPrep agarose (FMC) was inoculated with 2×10^6 primary transformants and allowed to solidify in an ice bath. Media was transferred to 30° and incubated for 48 h. Bacteria-containing media was then centrifuged and the resulting cells were titered as above and frozen in 2x LB containing 12.5% glycerol.

Western blot analysis of library proteins

Protein expression from clones was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) (1 mM) to exponentially growing cultures and harvesting cells 4 h later. Cells were lysed in buffer containing 50 mM KH₂PO₄, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–16% NuPAGE gels (Invitrogen) and visualized by staining with Lumio Detection Reagents (Invitrogen). Lysates were transferred to polyvinylidene fluoride (PVDF) membrane for western blot analysis.

The following antibodies were used: TROMA (rat anti-mouse CK19) (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA), rabbit anti-HNF4 (Assay Biotech, Sunnyvale, CA, USA), and rabbit anti-OST (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were goat anti-rat IgG-HRP (AbD Serotec, Raleigh, NC, USA) and goat anti-rabbit IgG-horse radish peroxidase (HRP) (Invitrogen). Bands were visualized using Western Lightning Plus ECL reagents (Perkin Elmer, Waltham, MA, USA).

DNA sequencing and mass spectrometry analysis of library clones

Individual NMC library clones were grown in LB media containing carbenicillin (50 µg/mL). Library proteins were isolated for mass spectrometry analysis by 4 h of IPTG induction of individual clones. Lysates were separated on a 4–16% NuPage gel and visualized with Lumio Detection reagents as above. Fluorescent bands were dissected from the gel and analyzed by the UC Denver Cancer Center Mass Spectrometry core facility. Plasmid DNA was purified using the QiaPrep kit (Qiagen, Valencia, CA, USA) and sequenced using a T7 promoter primer by the UC Denver Cancer Center DNA sequencing core facility.

RESULTS

Assessment of cholangiocyte cDNA library quality

An expression cDNA library derived from a normal mouse cholangiocyte line was generated and assayed for average insert size and range of expressed protein size. Following restriction enzyme digestion of 35 individual clones, an average cDNA insert of 1.17 kb was determined with the insert size ranging from 0.3–3.5 kb. Eight representative samples are shown in Figure 1. Further analysis of the library was performed on bacterial cell lysates post-induction of protein expression by IPTG (Fig. 2). Fluorescent proteins were produced ranging in size from 10–100 kD. In order to determine if adequate coverage of the cholangiocyte transcriptome was present, a titer of the primary library was performed. It was determined that 4.5×10^6 independent clones were present ensuring greater than 100-fold coverage of genes expressed at 0.01% of the total transcript pool.

Determination of cDNA library content

Before the cDNA library generated can be used in subsequent studies, it must first be verified that the proteins expressed by it are indeed cholangiocyte-derived. Three different methods were used for this aim. First, western blot analysis was performed using antibodies to proteins known to be expressed in bile ducts. Figure 3 demonstrates the presence of four such proteins: cytokeratin 19 (CK19),¹⁶ Annexin A1,¹⁷ hepatocyte nuclear factor 4a (HNF4a),¹⁸ and organic solute transporter (Ost).¹⁹ All of these proteins are expressed in the parental cell line for the library, NMC, and in the library post-transcriptional induction with IPTG. For CK19 and Ost, expression is shown in the absence of induction, indicating that expression is leaky in some instances. A second method used to determine proteins present in the library is mass spectrometry of protein products from individual cDNA clones. Individual bacterial colonies were grown in the presence of IPTG, bacterial lysates were separated by SDS-PAGE and fluorescent proteins were isolated and sequenced (Fig. 4). The last identification method used was direct DNA sequencing of individual cDNA-containing plasmids using a T7 promoter primer (data not shown). A summary of all proteins identified by the above methods is shown in Table 1. All but three of the revealed proteins have had confirmed expression in mouse cholangiocytes or total liver preparations. The three undetermined proteins have not been tested for expression in liver.

DISCUSSION

This investigation led to the generation of a cholangiocyte-specific expression cDNA library. SEREX analysis has become an important tool in identifying autoantigens targeted in both spontaneous and induced autoimmune disease in rodents and humans.^{13,34,35} One such potential disease for study using this newly created expression cDNA library is the mouse model of BA. As work performed in our laboratory has established the presence of antibodies to at least one known autoantigen in this model,⁷ the generation of a panel of potential novel autoantigens for SEREX screening would be advantageous. To this end, we successfully constructed a cDNA library expressing mouse cholangiocyte antigens *in E. coli*. The size of the library, as measured by number of independent clones, ensures that the spectrum of proteins contained within is fully reflective of the cholangiocyte cell line. Presence of both cholangiocyte-specific and non-specific proteins has been confirmed by a variety of methods and this library is available for screening with autoantibody-containing serum. In addition, autoreactive T-cell clones may be screened for potential reactivity with proteins present in the library with the goal of identifying T-cell autoantigens in murine BA. Identification of antigens relevant to murine BA is a stepping stone to deciphering autoimmune mechanisms of biliary tract injury in human BA.

It is important to note, that as this library is derived from normal mouse cholangiocytes, this library will be of use to the study of the autoimmune response in many murine biliary disease models, including PBC, PSC and autoimmune cholangitis.^{36–38} This opportunity to identify and characterize disease-relevant protein targets in a number of cholangiopathies may lead to insight into global mechanistic questions related to autoimmune-mediated liver diseases.

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Figure 1. Molecular weight analysis of cDNA plasmid inserts. DNA from individual cDNA clones was subjected to restriction enzyme digestion using *BsrGI*. Samples were visualized on an agarose gel. The arrow indicates the pDONR222 plasmid at 2.5 kb. Other bands are cDNA inserts. MW-1kb plus marker (Invitrogen, Carlsbad, CA, USA).

Figure 2. Molecular weight distribution of expressed proteins from cholangiocyte cDNA library. Bacterial cells containing cDNA clones were grown for 4 h in the present of IPTG. Resulting cell pellets were lysed and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1. Protein molecular weight marker with sizes (in kD) indicated. Lane 2. Library at time 0. Lane 3. Library at 4 h.

Figure 3.

Western blot analysis of cholangiocyte-derived cDNA library. Protein expression from the cDNA library was induced by 4 h in culture with 1 mM IPTG. Antibodies to cytokerafin 19 (a), Annexin A1 (b), HNF4a (c), and Ost (d) were tested. The arrows next to each panel represent the specific target antigen. Lane 1 is time 0. Lane 2 is 4 h without IPTG, and lane 3 is 4 h with IPTG. Lane 4 is the parental cell line (normal mouse cholangiocyte cell line, NMC).

Figure 4.

Representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel of individual cDNA clones. Individual bacterial colonies were grown in the presence of 1 mM IPTG, lysed and separated by SDS-PAGE. Fluorescent proteins were visualized and cut from the gel (white boxes). Peptides were purified from gel slices and sequenced via mass spectrometry. Molecular weight marker is designated in kD.

Table 1

Known proteins identified in the cholangiocyte library

Protein	Method of ID	Expression in liver/bile duct	References
Cytokeratin 19	WB, MS	Y	16
Annexin A1	WB	Y	17
HNF4a	WB	Y	18
Ost	WB	Y	19
Galectin-3	MS	Y	20
Cytokeratin 18	MS	Y	21
RNA U export adaptor protein	MS	Y	22
DEPD6 mTOR-interacting protein	MS	Y	23
Amyloid-like protein 1	MS, PS	Y	24
Heat shock protein 90	MS, PS	Y	25
60s ribosomal protein L5	MS, PS	Y	26
Bicc1, bicaudal C protein	PS	Y	27
Ogfod 1	PS	Y	28
Cytochrome c oxidase subunit Vic	PS	Y	29
Cdc42	PS	Y	30
Eef1a1 translation elongation factor	PS	Y	31
Cytochrome oxidase subunit III	PS	Y	32
ADP-ribosylation factor interacting protein 2	PS	Y	30
SenP5, SUMO protease	PS	Y	33
Surf1, mitochondrial protein	PS	Y	30

ADP, adenosine diphosphate; HNF4A, hepatocyte nuclear factor 4a; MS, mass spectrometry; PS, plasmid sequencing; WB, western blot.