Screening of augmenter of liver regeneration-binding proteins by yeast-two hybrid technique

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OBJECTIVE: To investigate the biological function of augmenter of liver regeneration (ALR), we used yeast-two hybrid technique to detect proteins in hepatocytes interacting with ALR.

METHODS: ALR bait plasmid was constructed by using yeast-two hybrid system 3, then transformed into yeast AH109. The transformed yeast was mated with yeast Y187 containing liver cDNA library plasmid in a 2 × YPDA medium. Diploid yeast was plated on a synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade) containing x-α-gal for selection and screening. After extracting and sequencing of the plasmid from blue colonies. Analysis was performed by bioinformatics.

RESULTS: Of 36 colonies sequenced, 14 are metallothionein, 12 albumin, and 3 selenoprotein P. One colony is a new gene with unknown function.

CONCLUSION: The successful cloning of gene of ALR interacting protein has paved the way for studying the physiological function of ALR and associated proteins.

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Key words: augmenter of liver regeneration; screen; yeast-two hybrid

Introduction

Augmenter of liver regeneration (ALR) as a mitogen for hepatocyte mitosis and a key factor for hepatic stimulating substance plays an important part in the regulation of liver regeneration.[1,2] Its function is also related to the regeneration of other organs such as promoting the mRNA stability of both nuclear and mitochondria transcripts, especially for the actively proliferating cell types, for instance, sperm cells. The multiple functions of ALR are biophysiologically complicated, and await further study. In our study, we identified ALR-binding proteins by yeast-two hybrid technique. With the counterpart of the ALR-binding protein, we tried to detect the mechanism in which ALR functions.

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Methods

Strain and reagents

E. coli DH5α (SupE44 △U169 (Φ80 lacZ△M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was kept in our laboratory. Others included yeast cell AH109 and plasmid vector pGBKT7, yeast YPD medium, SD/-Trp medium (Clontech Co., USA); pGEM-T vector, acrylamide and bis-acrylamide, isopropylthio-β-D-galactoside IPTG, X-β-gal (Promega Co., USA); Taq DNA polymerase, T4-DNA ligase, EcoR I and Pst I (Takara Co., Japan); lithium acetate, adenine, 3, 3'-diaminobenzidine

DAB (Sigma Co., USA); DNA purification with glass milk kits (BioDev Co., China); PCR primer synthesized and gene sequencing (Shanghai Bioasia Co., China).

Construction of pGBKT7-ALR expression vector

The ALR DNA fragment purified with DNA purification kits was ligated into the pGEM-T vector (the resulted vector has been designated as pGBKT7-ALR) by T4-DNA ligase at 16 ℃ overnight and transformed into *E. coli* DH5α by $CaCl_2$ perforation. *E. coli* DH5α containing the ALR PCR product was grown on a luria-bertani broth plate containing ampicillin (100 mg·L^{-1}), IPTG (1 mmol·L^{-1}), and X-β-gal (20 μg·μl^{-1}). White colonies were selected and incubated at 37 ℃, and shaked 250 r·min^{-1} for over 12 hours. Plasmid DNA was extracted for identification by digestion with EcoR I and Pst I and sequence analysis. Plasmid pGBKT7 was digested with EcoR I and Pst I and ligated with the digested ALR fragment in the plasmid DNA, then introduced into *E. coli* DH5α and grown on a luria-bertani broth plate containing kanamycin (50 mg·L^{-1}). Colonies were selected randomly and identified by the same method.

Since the c-myc tag was contained in the expression vector pGBKT7 ahead of the multi-cloning site, the expressed ALR protein also contained c-myc. We detected this product by immunological reaction with the monoclonal antibody of c-myc.

Yeast cell mating experiment

The expressive vector pGBKT7-ALR was used to transform competent yeast cells AH109,[3] while the expression of ALR was confirmed by Western blot hybridization. The AH109 yeast cells were cultured to 1×10^9·ml^{-1}, and mixed with yeast cells containing hepatocyte cDNA library at 1×10^{11}. Mating experiment was conducted overnight at 30 ℃ and shaked slightly. Twenty-four hours later, the mated yeast cells were plated on 25 plates of SD/-Trp/-Leu/-His and 25 plates of SD/-Trp/-Leu/-His/-Ade. After growing for 6-18 days, the colonies with a diameter of more than 2 mm were picked up for the examination of expression of α-galactosidase on QDO plates with X-α-gal. The emerged blue colonies on QDO plates were identified positive.

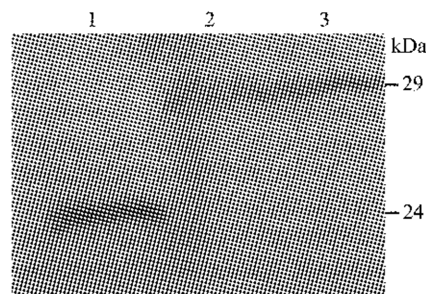

Fig. 1. Western blot hybridization of fusion protein of ALR-c-myc by pGBKT7-ALR transformed yeast cells. Lane 1: pGBKT7 positive control; Lanes 2 and 3: fusion protein expressed by pGBKT7-ALR.

Plasmid DNA extraction and DNA sequencing and analysis

The plasmid DNA was extracted by Lyticase method according to the product manual, and used to transform JM109 *E. coli* by electroporation;[4] and the ampicilin-resistant colonies were picked up for restriction mapping analysis and DNA sequencing. The plasmid DNA was also used in backcross experiment, i.e. the plasmid DNA was used to transform Y187 yeast cells and mated to AH109 yeast cells containing pGBKT7-ALR. The mated yeast was plated on the QDO plate. The DNA sequence was searched for homologous DNA sequences in GenBank by BLAST tool.

Results

Expression of ALR in yeast cells

The transformed AH109 with pGBKT7-ALR expressed ALR as the fusion protein with human c-myc tag. The expressed fusion protein could be probed with monoclonal antibody against human c-myc (Fig. 1). The positive control was the yeast cells expressing c-myc tag containing a DNA-binding domain $Gal4_{1-147}$. The molecular weight for c-myc tag was about 18.2 kDa, and for the fusion protein, about 33.2 kDa.

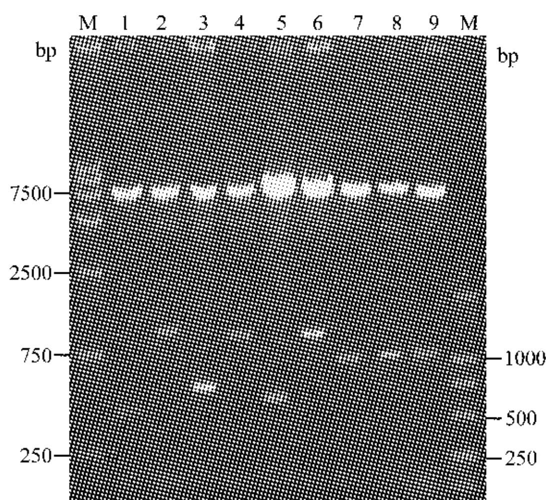

Fig. 2. Restriction mapping of clonies 1-9 by Bgl II digestion.

Restriction mapping of partial positive colonies

There were two Bgl II sites flanking the cloning site of pGADT7 vector, so the DNA insert could be released by Bgl II digestion. Nine positive clonies were picked up for restriction mapping analysis. Agarose electrophoresis results indicated that each colony contains a DNA insert with different size (Fig. 2).

DNA sequencing results

Altogether 36 colonies were picked up for DNA sequencing analysis (Table). Only one DNA fragment was identified as a new gene with unknown functions, and other 35 DNA fragments have homology sequences in GenBank.

Table. ALR-binding proteins identified by yeast-two hybrid technique

No.	Name of homology gene	Clone numbers identified	Homology (%)
1	Metallothionein	14	100
2	Albumin	12	100
3	Selenoprotein P	3	98
4	NADP dehydrogenase	2	100
5	Complete factor H related 3	1	99
6	Transitional endoplasmic reticulum ATPase	1	99
7	Elongation factor 1-α	1	99
8	Carboxypeptidase N 83 kDa chain	1	96
9	Unknown gene	1	98

Discussion

The ALR-coding gene is conserved in all species from yeast to mammalians, even in some kinds of DNA viral genomes ALR-like genes could be found, indicating that ALR superfamily plays an important role in the evolution process.[5] Human ALR, 40% homology to the ERV1 gene in yeast, holds the same function of oxidation/phosphorylation, generation of mitochondria, and cell cycle regulation as scERV1 in the yeast cells.[6,7] Mammalian ALR is a key factor for the regulation of liver regeneration in mammalians,[2,18] including liver failure caused by toxin,[8] transplanted pancreatic islet cells in muscles,[9] and development of other organs.[10] The recombinant ALR is also a potential drug for the promotion of liver regeneration in clinical use. Although the biochemical drugs containing ALR have been used widely with gratifying results,[11] their mechanism remains obscure. As a growth factor, ALR may affect via a receptor on the hepatocyte membrane,[12] and transduce its signal through protein kinase C (PKC), and finally stimulate the synthesis of DNA in the cell nucleus. The ALR-binding protein identification may give a clue to the study of the mechanism of recombinant ALR. Previous studies[13,14] indicated that recombinant ALR may stimulate liver regeneration via inhibiting the activity of natural killer (NK) cell activity, whereas other indicated that ALR may sti-mulate liver regeneration via regulating the functions of other liver regeneration-related factors.[7] In order to clarify the molecular mechanism involved in the liver regeneration regulation, we screened ALR-binding proteins by yeast-two hybrid technique.

The yeast-two hybrid system 3 is a key technique established by Clontech Co., USA for the identification of protein-protein interaction,[15-17] by type a and α mating mechanism. The proteins expressed by bait plasmid and cDNA plasmid in the diploid yeast cells could form protein-protein complex and could be selected by dropout medium and

α-galactosidase activity measurement. The 3 reporter gene expressions plus α-galactosidase selection may avoid a false positiveness of 95%. We obtained 36 colonies from the screening process, and the DNA sequencing analysis indicated that 35 colonies are homology to at least one gene in the GenBank database, and only one is not homology to any known genes presently, indicating a new gene unidentified. Among them, the ALR-binding proteins such as 14 clonies of metallothionein, 12 clonies of albumin may be related to the transportation process of ALR protein in the circulation. Selenoprotein P, an anti-oxidation protein, may be related to the function of ALR. Other ALR-binding proteins such as elongation factor 1-α, transitional endoplasmic reticulum ATPase, carboxypeptidase N 83 kDa chain, complete factor H related 3, and NADP dehydrogenase, play a very important role in energy metabolism. The unknown gene identified in this study is still under investigation in this laboratory.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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