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Molecular characterization and functional analysis of a protease-related protein in Chang-liver cells

Congrui Wang[1], Huiyong Zhang[2], Huigen Feng[2], Baosheng Yang[2], Jogenananda Pramanik[1,3], Zhikun Guo[4] & Juntang Lin[2,4,5,]*

Departments of [1]Biochemistry and Molecular Biology, [2]Life Science and Technology, Xinxiang Medical University, 453003 Xinxiang, China, [3]Allianze College of Medical Sciences, Waziria Medical Square, Kepala Batas 13200, Pinang, Malaysia, [4]Key Open Laboratory for Tissue Regeneration of Henan Universities, Xinxiang Medical University, 453003 Xinxiang, China, [5]Institute of Anatomy I, School of Medicine, University of Jena, Teichgraben 7, 07743 Jena, Germany

In this study, the cDNA library of Chang-liver cells was immunoscreened using common ADAMs antibody to obtain ADAM related genes. We found one positive clone that was confirmed as a new gene by Blast, which is an uncharacterized helical and coil protein and processes protease activity, and named protease-related protein 1 (*ARP1*). The submitted GenBank accession number is AY078070. Molecular characterizations of *ARP1* were analyzed with appropriate bioinformatics software. To analyse its expression and function, *ARP1* was subcloned into glutathione S-transferase fusion plasmid pGEX-2T and expressed by *E. coli* system. The *in vitro* expression product of *ARP1* was recognized by common ADAMs antibody with western blot. Interestingly, ARP1 cleaves gelatine at pH9.5, which suggests it is an alkaline protease. Semi-quantitative RT-PCR result indicates that ARP1 mRNA is strongly transcribed in the liver and the treated Chang-liver cells. [BMB reports 2010; 43(5): 375-381]

INTRODUCTION

Chang-liver cell was a cell line generated from human liver cancer tissue, which was often used in scientific research (1-3). After Chang-liver cells treated by the modified Hanks buffer containing ATP, compared to untreated control cells, there were new proteins recognized by ADAMs antibody, and some of them possessed metalloprotease activity (4).

Metalloproteases are a superfamily of protease including ADAMs (a disintegrin and metalloproteases) etc. (5). This superfamily occupy important roles in normal or abnormal physiological conditions, such as proliferation, cell motility, remodelling, wound healing, angiogenesis, and key reproductive events (6). Metalloproteases also play roles in liver regeneration and fibrosis (7, 8). Among metalloproteases, ADAM family belong to type I transmembrane proteins, which and their related proteins are potentially involved in proteolysis, adhesion, cell fusion and cell signalling (9, 10).

To study the ADAMs or their related proteins involved in Chang-liver cells under abnormal conditions, such as being treated by ATP in modified Hanks buffer, it is necessary to clone the full-length cDNAs of them, for example, to screen the target gene from cDNA library (11, 12). We constructed the cDNA expression library of Chang-liver cells treated by the modified Hanks buffer containing ATP (13), then screened the cDNA library with common ADAMs antibody raised against a conserved region from the metalloprotease domain of several known ADAMs molecules and predicted to recognize most ADAMs and ADAM related proteins. A new gene named protease-related protein 1 (*ARP1*) was obtained from the cDNA library (4, 13).

In the present study, we analyzed the molecular characteristics of *ARP1* and investigated its function *in vitro*. *ARP1* is located on human genome 16q22.1. It may be a secreted protein with a potential signal peptide. Glutathione S-transferase (GST)-ARP1 fusion protein was obtained by *E. coli* expression system. Protease activity assay analysis revealed that ARP1 strongly sheds gelatine at pH9.5, which suggests it has alkaline protease activity. Our results may provide the basis to further investigate the roles of ADAMs or protease related genes.

RESULTS AND DISCUSSION

Immunoscreening for cDNA library of Chang-liver cells

We have investigated the molecular alteration of Chang-liver cells treated by Hanks buffer containing ATP, and found that new ADAMs or their related proteins are induced after ATP treatment (4). The cDNA library of Chang-liver cells treated by the modified Hanks buffer containing ATP was also constructed (13). This cDNA library was immnunoscreened with common ADAMs antibody (Fig. 1A-C). After immunoscreening and sequencing, we obtained a series of positive clones from

*Corresponding author. Tel: 49-3641-938546; Fax: 49-3641-938512; E-mail: juntang.lin@googlemail.com

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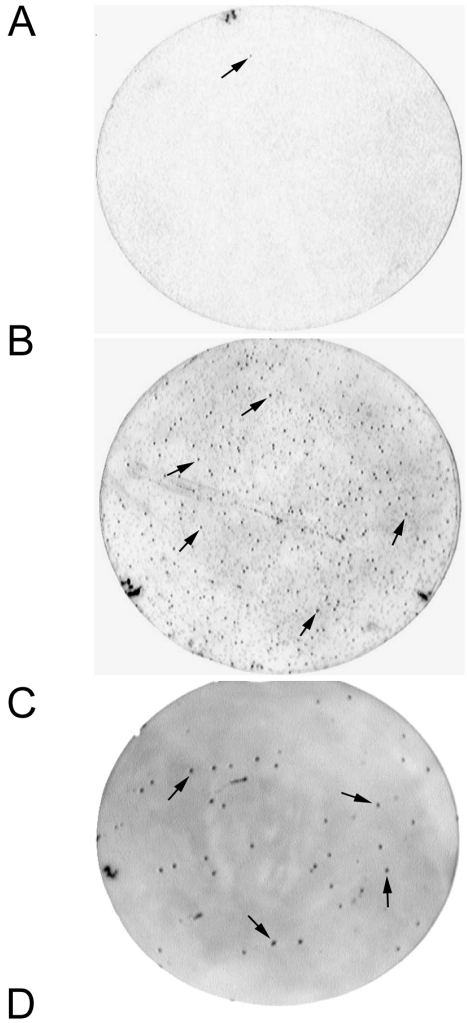

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Clone No.	Homologous gene	Similarity	GenBank No.
CLT-A-1	Homo sapiens proteinase-related protein mRNA (ARP1), complete cds	100%	AY078070
CLT-A-2	Homo sapiens, clone IMAGE:3510338, mRNA	100%	BC008007
CLT-A-3	Homo sapiens, clone IMAGE:3856788, mRNA	99%	BF032695
CLT-A-4	Homo sapiens A kinase (PRKA) anchor protein (yotiao) 9, mRNA	98%	BC015533
CLT-A-5	Homo sapiens N-ethylmaleimide-sensitive factor (NSF), mRNA	98%	NM_006178
CLT-A-6	Homo sapiens ADAM metallopeptidase domain 10 (ADAM10), mRNA	100%	NM_001110
CLT-A-7	Homo sapiens ADAM metallopeptidase domain 15 (ADAM15), T1, mRNA	100%	NM_207191
CLT-A-8	Homo sapiens, clone MGC:19844 IMAGE:2939726, mRNA, complete cds	96%	BC011869
CLT-A-9	Homo sapiens ADAM metallopeptidase domain 17 (ADAM17), mRNA	99%	NM_003183

Fig. 1. Gradient immnunoscreening of positive clones from the cDNA expression library with common ADAMs antibody. (A) positive dots of phage clone from first cycle of screening by common ADAMs antibody. The original cDNA library was diluted in 1 : 1,000. Arrow indicates the positive plaque. (B) positive dots of phage clone from second cycle of screening with the dilution (1 : 100) of the positive plaque from the first screening. (C) positive dots of phage clone from third screening with the dilution (1 : 100) of the positive plaque from the second screening. Arrows show the positive plaques. The immunoscreening method performed was same for the three cycles. (D) the list of obtained positive clones including ARP1 from the cDNA expression library screened with common ADAMs antibody.

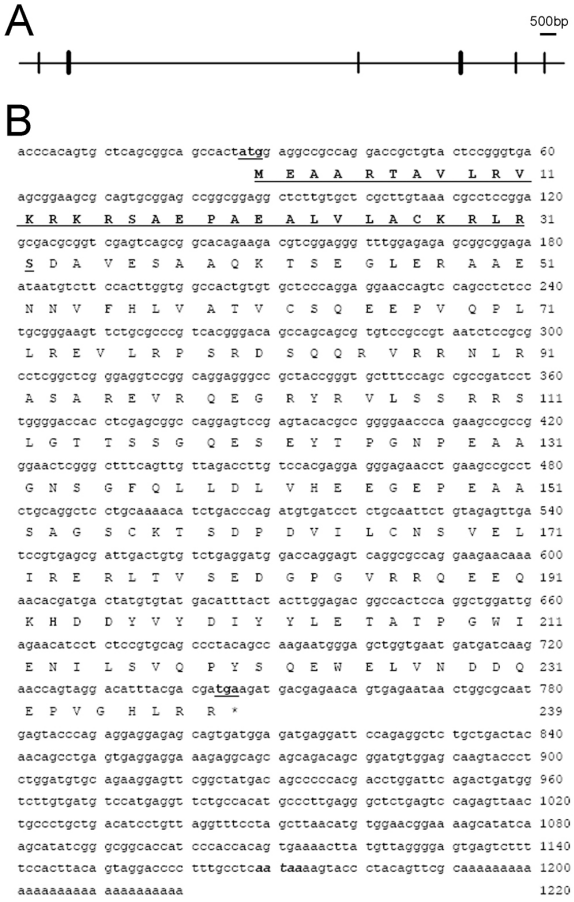

Fig. 2. The schematic presentation of *ARP1* gene structure, as well as *ARP1* cDNA sequence and the deduced amino acids. (A) the schematic presentation for the composition of *ARP1* gene, which consists of 6 exons and 5 introns. The vertical shows exons, and the line between each two adjacent exons shows intron. (B) the cDNA sequence of *ARP1* and its reduced amino acids from the putative ORF. The boldfaced and underlined atg and tga are the start and stop codens, respectively. The boldfaced and Italic sequence (*aataa*) is the polyadenylation signal sequence. The putative signal peptide sequence is boldfaced and underlined.

the cDNA library (Fig. 1D). One of them (CLT-A-1) shows strong immunoreactivity with common ADAMs antibody, named ARP1. The length of *ARP1* is 1,220 bp with polyA+, encoding 239 amino acids (aa, Fig. 2).

Bioinformatics analysis of ARP1

Sequence analysis indicates that *ARP1* possesses 6 exons and 5 introns (Fig. 2A). The cDNA of *ARP1* possesses a 720 bp open reading frame (ORF) from 27 bp to 746 bp, except for a 26 bp 5' untranslated region (UTR) and a 474 bp 3' UTR. In the 3' UTR, there exists a typical polyadenylation signal aataa (14) and a long polyA+ tail (Fig. 2B), which indicates we obtained the full-length cDNA of *ARP1* from the constructed

cDNA library. The obtained sequence was analyzed using the BLAST program in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). The full-length cDNA of *ARP1* was submitted as GenBank accession no. AY078070.

The result from protein blast with blastp program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic tree analysis revealed that ARP1 amino acid sequence does not show high similarity with the members of ADAM family, but shows high homology with the uncharacterized proteins from human and other species (Fig. 3A). For example, ARP1 shares 99% (231/233) identities and positives with the unnamed protein product or solute carrier protein family member from *Homo sapiens* (accession no. BAB14538) (15, 16). ARP1 also shares high level identities and positives with other species, such as the unnamed protein product from *Macaca fascicularis* (94% identities and 96% positives, accession no. BAE91241) (17), the predicted hypothetical protein XP_848670 from *Canis familiaris* (79% identities and 84% positives, accession no. XP_853763), the liver regeneration-related protein from *Rattus norvegicus* (73% identities and 81% positives, accession no. EDL92442.1) (18), and the unnamed protein product from *Mus musculus* (72% identities and 81% positives, accession no. BAE27395) (19). The above results further confirmed that human ARP1 is an uncharacterized protein. Interestingly, human ARP1 shares high similarity with rat liver regeneration-related protein. Liver tissue could regenerate after damage or clinical operation in mammals (20). Tissue regeneration is an extremely complicated procedure involving cell differentiation, dedifferentiation and redifferentiation (21). Human ARP1 comes from Chang-liver cells after the treatment with ATP which may result in cell dedifferentiation or redifferentiation (13), so human ARP1 may be involved in liver regeneration through involvement in cell differentiation and redifferentiation.

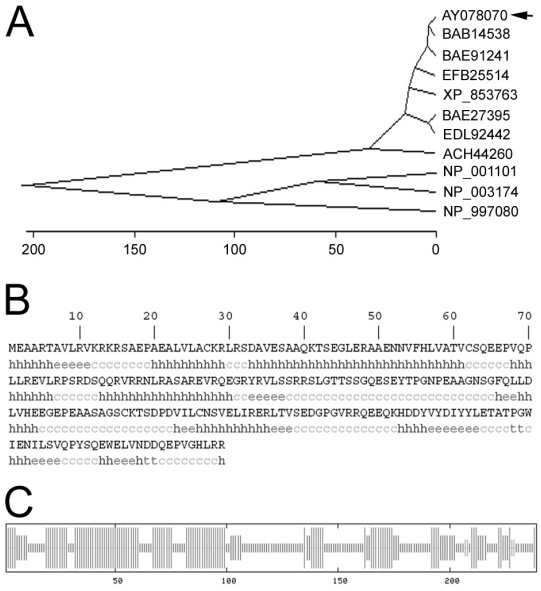

Fig. 3. The phylogenetic tree and the predicted secondary structure of ARP1 from SOPMA. (A) the phylogenetic tree generated among the proteins which share similarities with ARP1. AY078070, protease-related protein 1 (ARP1); BAB14538, human unnamed protein; BAE91241, *macaca fascicularis* unnamed protein product; EFB25514, hypothetical protein PANDA_012377; XP_853763, *canis familiaris* predicted hypothetical protein XP_848670; BAE27395, mouse unnamed protein product; EDL92442, rat liver regeneration-related protein; ACH44260, *taeniopygia guttata* hypothetical protein; NP_001101, human ADAM10 metalloprotease domain; NP_003174, human ADAM17 metalloprotease domain; NP_997080, human ADAM15 metalloprotease domain. The numbers below indicate the amino acid difference between the various protein sequences. (B) the linearization structure of ARP1 protein. h, alpha helix; c, random coin; e, extended strand; t, beta turn. (C) the secondary structure of ARP1 protein shown in the schematic presentation. Alpha helix and extended strand were denoted as vertical long bars and vertical short bars, respectively, with the horizontal line presenting the random coil running through the whole molecule.

Analysis with SOPMA program shows the secondary structure of ARP1 to be composed of 43.10% random coil (c), 42.26% alpha helix (h), while 12.97% extended strand (e) and 1.67% beta turn (Fig. 3B). Alpha helices are located within the main body especially in the N-terminal region of ARP1, while random coils mainly penetrate through the rest part of this sequence accompanied by a small amount of alpha helixes and extended strands, as well as a few beta turn (Fig. 3B, C). This indicates that ARP1 is a predominantly helical and coil protein. The comparative modelling of three-dimensional (3D) structure was performed using SWISS-MODEL and HHpred programs. There is no existing model which shows high similarity with the 3D structure of ARP1 since it is a totally uncharacterized protein. It may be an interesting point to investigate the high level structure of ARP1 later.

Human genome blast revealed that *ARP1* is located on the long arm of human chromosome 16 (16q22.1, Fig. 4A). Its molecular weight is 26.8kD, and the isoelectric point (pI) is 5.21. Signal peptide analysis revealed a most likely cleavage site between positions S32 and D33 (Fig. 2B), which suggests that the putative ARP1 protein possibly possesses a signal peptide of 32 aa at the N terminus.

Fusion protein expression of ARP1 by *E. coli*

The existence of *ARP1* has been confirmed by PCR with the common primers from the cDNA library kit running by 1.2% agarose gel and sequencing (Fig. 4B). Furthermore, another PCR was performed to amplify the ORF of *ARP1* with primers containing *Bam*HI or *Eco*RI enzyme site, respectively. The PCR product for the ORF of *ARP1* digested doubly by *Bam*HI and *Eco*RI was inserted into the expression vector pEGX-2T which was digested by the same enzymes. The recombinant plasmid was named pEGX-2T-*ARP1*.

pEGX-2T-*ARP1* was used to transform *E. coli* cell BL21 for protein expression *in vitro*. After induction with isopropyl-b-D-thiogalactoside (IPTG), the expression product was analyzed

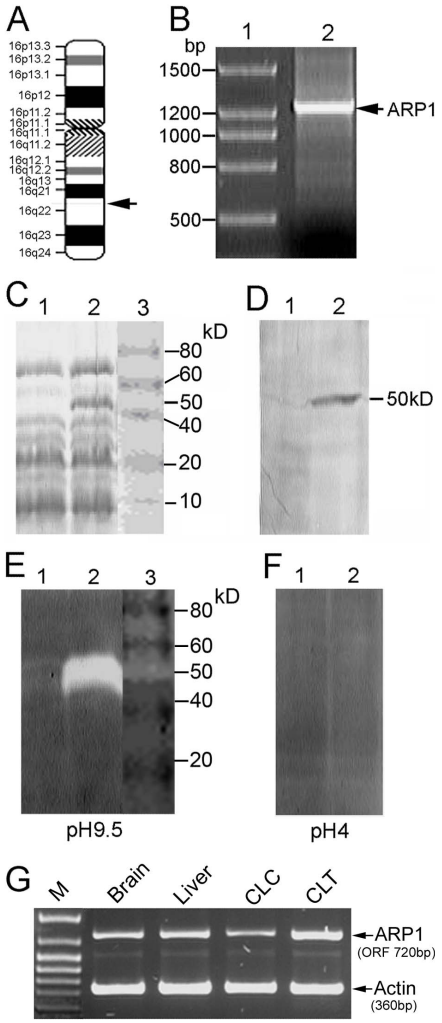

Fig. 4. Chromosome location, cDNA amplification, protein expression *in vitro*, function and expression profile analysis of human ARP1. (A) human *ARP1* is located on the long arm of chromosome 16 (16q22.1, arrow). (B) PCR amplification for the full-length cDNA of *ARP1*. Lane 1 indicates the marker, while lane 2 shows PCR product for the full-length cDNA of *ARP1*. (C) the GST-ARP1 fusion protein expressed from pEGX-2T-*ARP1* shown from SDS-PAGE (50 kD band). (D) confirmation of the expression product of pEGX-2T-*ARP1* by western blotting with common ADAMs antibody. (E) strong protease activity of ARP1 at pH 9.5. (F) negative protease activity of ARP1 at pH4. In Fig. 4C-F, lane 1 is for the control without IPTG induction, while lane 2 is for the expression product of pEGX-2T-*ARP1* induced by IPTG. Lane 3 in Fig. 4C and 4E is the protein marker also applied in Fig. 4D and 4F, respectively. (G) the expression profile of ARP1 (ORF of ARP1) in human brain and liver, as well as in Chang-liver cells detected by semi-quantitative RT-PCR. Human beta-actin (Actin) serves as an internal control. M, DNA marker; CLC, normal Chang-liver cells; CLT, Chang-liver cells treated with Hanks buffer containing ATP.

by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Compared to the uninduced control (lane 1 in Fig. 4C), there is an about 50 kD protein band after induction with IPTG (lane 2 in Fig. 4C). Since the expression product is GST fusion protein, and the molecular weight of GST is 26 kD (16), of ARP1 is 26.8 kD, the 50 kD band should be the fusion protein of ARP1 and GST which was predicted (Fig. 4C). The other control of the empty pEGX-2T induced by IPTG to express GST (26 kD protein) is not shown here. To further confirm the expression product is GST-ARP1 fusion protein, western blotting was performed with common ADAMs antibody (Fig. 4D). The result clearly shows that there is a 50 kD band after IPTG inducing expression (lane 1 in Fig. 4D), but there is no immunoreactive band in the control, the sample without IPTG induction (lane 2 in Fig. 4D). It indicates that we successfully obtained the expression product of ARP1 *in vitro*.

Alkaline protease activity of ARP1

Crude protein extract of GST-ARP1 fusion protein was used for the protease activity assay. Result from SDS-G-PAGE suggests that the recombinant protein of ARP1 is a pH-dependent protease which can shed gelatine *in vitro*. At pH9.5, the fusion protein ARP1 has strong protease activity (Fig. 4E). However, ARP1 does not shed gelatine at pH 4 (Fig. 4F). These results indicate that ARP1 is an alkaline protease, at least it can strongly shed gelatine at pH9.5 *in vitro*.

Based on the above analysis, ARP1 is still an uncharacterized protein. ARP1 should be a family member of ADAMs or ADAM related proteins because it was recognized by common ADAMs antibody. However, we failed to find high homology among ADAMs family and ARP1 (Fig. 3A). Since common ADAMs antibody was designed according to the conserved region from the metalloprotease domain of several ADAM members, it may also recognize some other proteases which have relation with ADAM family. So, we hereby concluded that ARP1 is an ADAM related protein, and named it protease-related protein based on its protease activity. The molecular confirmation and the functional investigation of ARP1 will provide the basis to further study its role in liver cancer or regeneration.

Expression profile of ARP1 in tissues and cells

To monitor the transcription of ARP1 in human tissues and cells, we performed semi-quantitative RT-PCR analysis using human brain, liver, normal Chang-liver cells (control, CLC) and Chang-liver cells with treatment (CLT in Fig. 4G). Human beta-actin (Actin) was used as an internal control. The results showed that ARP1 mRNA is weakly detected in normal Chang-liver cells, but it is transcribed most strongly in the treated Chang-liver cells with Hanks buffer containing ATP, which is identical with the above result that ARP1 was obtained from treated Chang-liver cells. ARP1 is expressed more strongly in the liver than in the brain, so it is of interest to investigate the role of ARP1 in normal and abnormal liver tissue.

MATERIALS AND METHODS

cDNA library construction

Chang-liver cells were cultured in 5.0% CO_2 incubator at 37°C. Cells were treated with the modified Hanks buffer containing 2 mg/ml ATP (4). Then the treated cells were collected by centrifugation. Isolation of total RNA was carried out according to the protocol of RNAease kit (Qiagen). The Chang-liver cell cDNA library was constructed according to the protocol of SMART cDNA library construction kit (Clontech) and package protein of λ phage (Stratagene) (13).

The chemical and regents used in this study are purchased from Sigma or Merck companies except for specific mention.

Immunoscreening

The cDNA library of the treated Chang-liver cells was screened with the common ADAMs antibody. The immunoscreening procedure has been described by Lin *et al*. (13). Finally, the positive clones was sequenced by Shanghai Shenggong Company. One of positive clones with identical polyA+ was named protease-related protein 1 (*ARP1*).

Bioinformatics analysis of *ARP1*

The full-length genomic sequence and chromosome location of *ARP1* was obtained by blasting against the human genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The number and length of exons and introns were also obtained by comparison with the human genome. The ORF and translation of the nucleotide sequence, the predicted pI and molecular weight of ARP1, as well as the phylogenetic tree among Arp1 and other similar proteins, were analyzed by DNAStar software. Signal peptide of ARP1 was determined with online SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Secondary structure of ARP1 was predicted by SOPMA program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) (22). The 3D structure prediction was performed with SWISS-MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html) and HHpred programs (http://toolkit.tuebingen.mpg.de/hhpred).

Recombinant plasmid construction

According to the results from the sequencing and bioinformatics analysis for *ARP1*, primers with appropriate restriction enzyme sites were designed for the ORF of *ARP1*. The forward primer is 5'-GTGGATCCATGGAGGCCGCCAGGAC-3', the underlined sequence showing the *Bam*HI restriction site; the reverse primer is 5'-ATGAATTCCTACTGTAAGTGGAAAAGAC-3', the underlined sequence showing the *Eco*RI restriction site. The PCR template harbouring *ARP1* was denatured for 5 min at 94°C, followed by 35 cycles of amplification (denaturing for 60 sec at 94°C, annealing for 60 sec at 55°C, and extension for 80 sec at 72°C). After double digestion with *Bam*HI and *Eco*RI, the ORF of *ARP1* was directionally subcloned into GST fusion expression vector pEGX-2T (Promega), named pEGX-2T-*ARP1*.

In vitro expression of ARP1 in *E. coli*

E. coli strain BL21 was transformed with the recombinant plasmid pEGX-2T-*ARP1* (23). From resulting colonies, a single colony was inoculated at 37°C in Luria-Bertani (LB) medium containing ampicillin (100 mg/L), with shaking (180 rpm) until the optical density OD_{600} reached about 0.6 OD. Then the culturing LB medium was separated into two bottles. One was cultured as a control, and the other was induced by adding IPTG to a final concentration of 1 mM for ARP1 induction expression. The culture was continued for another 8 hours. The expression level of the protein was assessed by analyzing total protein extract on 10% (w/v) SDS-PAGE followed by Coomassie Brilliant Blue R250 staining (18, 24).

Western blot

The recombinant protein GST-ARP1 was resolved on 10% (w/v) SDS-PAGE and electro-blotted onto the PVDF membrane with the wet transfer method according to a standard procedure (25). Then it was detected using common ADAMs antibody which was used for cDNA library immunoscreening. The steps followed were modified from http://www.westernblotting.org.

Protease active assay with SDS-G-PAGE

SDS-PAGE containing gelatine (SDS-G-PAGE) was performed for protease active assay according to the modified protocol (26). Briefly, 1% (w/v) gelatine solution was added into the polyacrylamide gel for SDS-G-PAGE. 200 μg samples were loaded for the lysate product from the cultured bacteria containing ARP1 (induced by IPTG) and for the control (without IPTG induction). After electrophoresis, the gel was washed in washing buffer (3.03 g Tris-base, 12 ml Triton X-100, in 500 ml sterile water, pH 6.5) at room temperature for 30 min. Then the gels were washed in sterile water for three times, 10 min for each time. Next, the gels were incubated in the reactivation buffer (0.1 M glycine, 5 mM $CaCl_2$) with different pH value (pH 4, pH 7 and pH 9.5, respectively) at 37°C for 24 hours. Finally, the gels were stained by Coomassie Brilliant Blue R250. The protein band with protease activity, which digested gelatine at appropriate pH value, would be expected to lack staining (white colour).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed using the One Step RT-PCR Kit (Qiagen). Human brain and liver total RNA were purchased from Clontech company. The first strand of cDNA was synthesized *in vitro* using the SuperScript First-Strand Synthesis System (Invitrogen). Human beta-actin (Actin) was used as an internal control to monitor the amount of RNA. The upper primer for Actin was 5'-CGTGCTGCTGACCGAGGCCCC-3' and the lower primer 5'-GCTCCTTAATGTCACGCACGATTTC-3' (expected product length of 360 bp). The primers for

ARP1 are same as above (excepted product length of 720 bp). To obtain clear cDNA bands of both ARP1 and Actin in one reaction, the ratio of the primer concentration between ARP1 and Actin was adjusted to 2 : 1. The RT-PCR reaction was performed first at 50°C for 30 min for reverse transcription and at 95°C for 15 min for denaturation, followed by 30 cycles of amplification (denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min). The PCR products were analyzed on a 2% agarose gel, which was stained with ethidium bromide and photographed under UV light.

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REFERENCES

1. Chang, R. S. (1978) HeLa marker chromosomes, Chang liver cells, and liver-specific functions. *Science* **199**, 567-568.
2. Mitsumoto, Y., Sato, K., Ohyashiki, T. and Mohri, T. (1986) Leucine-proton cotransport system in Chang liver cell. *J. Biol. Chem.* **261**, 4549-4554.
3. Kaviarasan, S., Ramamurthy, N., Gunasekaran, P., Varalakshmi, E. and Anuradha, C. V. (2009) Induction of alcohol-metabolizing enzymes and heat shock protein expression by ethanol and modulation by fenugreek seed polyphenols in Chang liver cells. *Toxicol. Mech. Methods.* **19**, 116-122.
4. Wang, C. R., Hou, D. Y., Feng, H. G., Yang, B. S., Xu, C. S. and Lin, J. T. (2010) Induction of new ADAM related proteins from treated human Chang-liver cells. *Mol. Biol.* **44**, 5.
5. Lu X., Lu, D., Scully, M. F. and Kakkar, V. V. (2007) Structure-activity relationship studies on ADAM protein-integrin interactions. *Cardiovasc. Hematol. Agents Med. Chem.* **5**, 29-42.
6. Amălinei, C., Căruntu, I. D. and Bălan, R. A. (2007) Biology of metalloproteinases. *Rom. J. Morphol. Embryol.* **48**, 323-334.
7. Dalakas, E., Newsome, P. N., Harrison, D. J. and Plevris, J. N. (2005) Hematopoietic stem cell trafficking in liver injury. *FASEB J.* **19**, 1225-1231.
8. Han, Y. P. (2006) Matrix metalloproteinases, the pros and cons, in liver fibrosis. *J. Gastroenterol. Hepatol. Suppl.* **3**, S88-91.
9. Black, R. A. and White, J. M. (1998) ADAMs: focus on the protease domain. *Curr. Opin. Cell. Biol.* **10**, 654-659.
10. Blobel, C. P. (2002) Functional and biochemical characterization of ADAMs and their predicted role in protein ectodomain shedding. *Inflamm. Res.* **51**, 83-84.
11. Abe, S., Koyama, K., Usami, S. and Nakamura, Y. (2003) Construction and characterization of a vestibular specific cDNA library using T7-based RNA amplification. *J. Hum. Genet.* **48**, 142-149.
12. Kim, K. S., Kim, H. W., Chen, T. T. and Kim, Y. T. (2009) Molecular cloning, tissue distribution and quantitative analysis of two proopiomelanocortin mRNAs in Japanese flounder (Paralichthys olivaceus). *BMB Rep.* **42**, 206-211.
13. Lin, J. T., Li, Y. C., Zhang, H. Y. and Xu, C. S. (2004) Construction of Chang Liver cDNA library and immunoscreening of ADAMs related genes. *Yi Chuan* **26**, 793-796.
14. Nguyen, N. Y., Suzuki, A., Cheng, S. M., Zon, G. and Liu, T. Y. (1986) Isolation and characterization of Limulus C-reactive protein genes. *J. Biol. Chem.* **261**, 10450-10455.
15. Strausberg, R. L., Feingold, E. A., Grouse, L. H., Derge, J. G., Klausner, R. D., Collins, F. S., Wagner, L., Shenmen, C. M., Schuler, G. D., Altschul, S. F., Zeeberg, B., Buetow, K. H., Schaefer, C. F., Bhat, N. K., Hopkins, R. F., Jordan, H., Moore, T., Max, S. I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A. A., Rubin, G. M., Hong, L., Stapleton, M., Soares, M. B., Bonaldo, M. F., Casavant, T. L., Scheetz, T. E., Brownstein, M. J., Usdin, T. B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S. S., Loquellano, N. A., Peters, G. J., Abramson, R. D., Mullahy, S. J., Bosak, S. A., McEwan, P. J., McKernan, K. J., Malek, J. A., Gunaratne, P. H., Richards, S., Worley, K. C., Hale, S., Garcia, A. M., Gay, L. J., Hulyk, S. W., Villalon, D. K., Muzny, D. M., Sodergren, E. J., Lu, X., Gibbs, R. A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Madan, A., Young, A. C., Shevchenko, Y., Bouffard, G. G., Blakesley, R. W., Touchman, J. W., Green, E. D., Dickson, M. C., Rodriguez, A. C., Grimwood, J., Schmutz, J., Myers, R. M., Butterfield, Y. S., Krzywinski, M. I., Skalska, U., Smailus, D. E., Schnerch, A., Schein, J. E., Jones, S. J., Marra, M. A. and Mammalian Gene Collection Program Team. (2002) Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 16899-16903.
16. Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R., Wakamatsu, A., Hayashi, K., Sato, H., Nagai, K., Kimura, K., Makita, H., Sekine, M., Obayashi, M., Nishi, T., Shibahara, T., Tanaka, T., Ishii, S., Yamamoto, J., Saito, K., Kawai, Y., Isono, Y., Nakamura, Y., Nagahari, K., Murakami, K., Yasuda, T., Iwayanagi, T., Wagatsuma, M., Shiratori, A., Sudo, H., Hosoiri, T., Kaku, Y., Kodaira, H., Kondo, H., Sugawara, M., Takahashi, M., Kanda, K., Yokoi, T., Furuya, T., Kikkawa, E., Omura, Y., Abe, K., Kamihara, K., Katsuta, N., Sato, K., Tanikawa, M., Yamazaki, M., Ninomiya, K., Ishibashi, T., Yamashita, H., Murakawa, K., Fujimori, K., Tanai, H., Kimata, M., Watanabe, M., Hiraoka, S., Chiba, Y., Ishida, S., Ono, Y., Takiguchi, S., Watanabe, S., Yosida, M., Hotuta, T., Kusano, J., Kanehori, K., Takahashi-Fujii, A., Hara, H., Tanase, T. O., Nomura, Y., Togiya, S., Komai, F., Hara, R., Takeuchi, K., Arita, M., Imose, N., Musashino, K., Yuuki, H., Oshima, A., Sasaki, N., Aotsuka, S., Yoshikawa, Y., Matsunawa, H., Ichihara, T., Shiohata, N., Sano, S., Moriya, S., Momiyama, H., Satoh, N., Takami, S., Terashima, Y., Suzuki, O., Nakagawa, S., Senoh, A., Mizoguchi, H., Goto, Y., Shimizu, F., Wakebe, H., Hishigaki, H.,

Watanabe, T., Sugiyama, A., Takemoto, M., Kawakami, B., Yamazaki, M., Watanabe, K., Kumagai, A., Itakura, S., Fukuzumi, Y., Fujimori, Y., Komiyama, M., Tashiro, H., Tanigami, A., Fujiwara, T., Ono, T., Yamada, K., Fujii, Y., Ozaki, K., Hirao, M., Ohmori, Y., Kawabata, A., Hikiji, T., Kobatake, N., Inagaki, H., Ikema, Y., Okamoto, S., Okitani, R., Kawakami, T., Noguchi, S., Itoh, T., Shigeta, K., Senba, T., Matsumura, K., Nakajima, Y., Mizuno, T., Morinaga, M., Sasaki, M., Togashi, T., Oyama, M., Hata, H., Watanabe, M., Komatsu, T., Mizushima-Sugano, J., Satoh, T., Shirai, Y., Takahashi, Y., Nakagawa, K., Okumura, K., Nagase, T., Nomura, N., Kikuchi, H., Masuho, Y., Yamashita, R., Nakai, K., Yada, T., Nakamura, Y., Ohara, O., Isogai, T. and Sugano, S. (2004) Complete sequencing and characterization of 21,243 full-length human cDNAs. *Nat. Genet.* **36**, 40-45.

17. Wang, H. Y., Chien, H. C., Osada, N., Hashimoto, K., Sugano, S., Gojobori, T., Chou, C. K., Tsai, S. F., Wu, C. I. and Shen, C. K. (2007) Rate of evolution in brain-expressed genes in humans and other primates. *PLoS Biol.* **5**, e13.
18. Florea, L., Di Francesco, V., Miller, J., Turner, R., Yao, A., Harris, M., Walenz, B., Mobarry, C., Merkulov, G. V., Charlab, R., Dew, I., Deng, Z., Istrail, S., Li, P. and Sutton, G. (2005) Gene and alternative splicing annotation with AIR. *Genome Res.* **15**, 54-66.
19. Carninci, P., Shibata, Y., Hayatsu, N., Sugahara, Y., Shibata, K., Itoh, M., Konno, H., Okazaki, Y., Muramatsu, M. and Hayashizaki, Y. (2000) Normalization and subtraction of cap-trapper-selected cDNAs to prepare full-length cDNA libraries for rapid discovery of new genes. *Genome Res.* **10**, 1617-1630.
20. Xu, C. S. and Chang, C. F. (2008) Expression profiles of the genes associated with metabolism and transport of amino acids and their derivatives in rat liver regeneration. *Amino Acids* **34**, 91-102.
21. Liu, H. X, Hu, D. H., Jia, C. Y. and Fu, X. B. (2006) Progress of cellular dedifferentiation research. *Chin. J. Traumatol.* **9**, 308-315.
22. Geourjon, C. and Deléage, G. (1995) SOPMA: significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments. *Cabios.* **11**, 681-684.
23. Zhang, Q., Yan, X., Zhang, L. and Tang, W. H. (2006) Cloning, Sequence Analysis, and Heterologous Expression of a β-Mannanase Gene from Bacillus Subtilis Z-2. *Mol. Biol.* **40**, 368-374.
24. Lin, L., Wang, X. and Wang, Y. (2006) cDNA clone, fusion expression and purification of the novel gene related to ascorbate peroxidase from Chinese wild Vitis pseudoreticulata in *E. coli*. *Mol. Biol. Rep.* **33**, 197-206.
25. Towbin, H., Staehelin, T. and Gordin, J. (1976) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354.
26. Xia, M., Xue, S. B. and Xu, C. S. (2002) Shedding of TNFR1 in regenerative liver can be induced with TNF alpha and PMA. *World J. Gastroenterol.* **8**, 1129-1133.