Identification of Hepatopoietin Interacting Proteins in Human Testis by Yeast Two-hybrid System*

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Abstract Hepatopoietin (HPO) has diverse functions in the regulation of cell growth and differentiation. Its high level expression in testis suggests that HPO may have important role for testicular physiological functions. Using HPO as "bait", a yeast two-hybrid library screen of human testis was performed. By screening and selecting the positive colonies, retesting interactions in yeast, amplifying the AD/library inserts, sequencing and sequence comparing, four HPO-interacting proteins were identified: NADH dehydrogenase 1, Na^+/K^+ ATPase beta-3 subunit (ATP1β3), phospholipase C delta 1 and epididymal secretory protein. The significance of identification of these proteins may provide mechanistic insight into the biologic role of HPO in testis.

Key words hepatopoietin, yeast two-hybrid, protein-protein interaction, human testis library

Hepatopoietin (HPO)/human augmenter of liver regeneration (ALR) is a novel hepatotrophic growth factor, which stimulates hepatocyte proliferation by two pathways. In the first, extracellular HPO triggers the MAPK (mitogen activated protein kinase) pathway by binding its specific receptor on the cell surface[1, 2], while in the second, intracellular HPO specifically modulates the AP-1 pathway through JAB1 (Jun activation domain-binding protein 1)[3]. HPO expression is not restricted to liver tissue[4, 5] and Alrp messenger RNA is synthesized in large amounts in the testis[6, 7]. Recently, we demonstrate that HPO is a flavin-linked sulfhydryl oxidase (SOX) but its native substrate is unclear. In addition, biochemical and immunohistochemical studies had indicated the important role of other sulfhydryl oxidases for testicular differentiation processes[8~10]. The proposed role of 15 ku HPO as a hepatotrophic growth factor is restricted to liver cells, while HPO, like other redox-active proteins and sulfhydryl oxidases, may have diverse functions in the regulation of cell growth and differentiation.

In order to get more information about the biologic role in testis and to find the native substrate of HPO, we used the full-length HPO (15 ku) fused to GAL4 DNA-binding domain as bait in the yeast two-hybrid system to screen a human testis cDNA library. Four HPO-interacting proteins were identified.

1 Materials and methods

1.1 Materials

1.1.1 Plasmid and strain: Yeast GAL4 activation domain (AD) vector pACT2, pGBKT7 DNA-BD vector, *Saccharomyces cerevisiae* host strain Y187 and yeast stain AH109 were from Clontech. *E. coli* DH5α and JM109 were from TaKaRa Biotechnology (Dalian) Co., Ltd.

1.1.2 Kits: Pretransformed MATCHMAKER human testis library (human testis cDNA library was cloned into pACT2 and pretransformed into Y187), MATCHMAKER two-hybrid system 3, YEASTMAKER™ yeast transformation kit and human testis mRNA library were from Clontech. Reverse transcription kit was from Invitrogen. DNA purification kit was from Promega.

1.1.3 Enzymes and other reagents: X-α-Gal, YPD, SD/-Trp, SD/-Leu, SD/-Trp/-Leu, SD/-Trp/-Leu/-His, SD/-Trp/-Leu/-His/-Ade were from Clontech. Glass bead and adenine were from Sigma. Taq DNA polymerase, Pyrobest DNA Polymerase with GC buffer and dNTP were from TaKaRa Biotechnology (Dalian) Co., Ltd. Restriction endonucleases *Eco*R Ⅰ, *Bam*H Ⅰ, *Hae* Ⅲ and T4 DNA ligase were from Promega. Anti-HPO polyclonal antibody was generated against bacterially produced recombinant protein. Western blotting Luminol Reagent was from Santa Cruz Biotechnology. Peroxidase-conjugated anti-rabbit IgG was from Jackson Immuno Research Laboratories, Inc. Hybond-P membrane was from Amersham Pharmacia Biotech.

1.2 Methods

1.2.1 Plasmid construction: Primer 1 (5′ CCG GAA TTC GCC TTT GTT CGG AGA AT 3′) as the forward primer with an *Eco*R Ⅰ site (underlined) and primer 2 (5′ CGC GGA TCC G GTC ACA GGA GCC ATC CTT 3′) as the reverse primer with a *Bam*H Ⅰ site (underlined) were designed for amplifying DNA

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fragment encoding HPO (390 base pairs, GenBank AF124603) from human testis cDNA library. The human testis cDNA library was generated from human testis mRNA library by performing a reverse transcriptional assay. The amplified DNA fragment was inserted into the DNA-BD vector pGBKT7 and transformed into *E. coli* JM109. The plasmid pGBKT7-HPO was verified by analysis of restriction digestion and DNA sequencing (BIOASIA Co., Shanghai). Primers were also synthesized by BIOASIA.

1.2.2 Testing the bait plasmid: pGBKT7-HPO was transformed into yeast strain AH109 according to the standard protocol by using the yeast transformation kit. Western blot was performed to identify the GAL4 DNA-BD/HPO from the transformants with anti-HPO polyclonal antibody. The DNA-BD/HPO protein was also tested for the possibility of auto-transcriptional activation according to the MATCHMAKER library user manual.

1.2.3 Screening the pretransformed human testis library by yeast mating: AH109-HPO strain was cultured in SD/-Trp selection liquid media overnight. The overnight AH109-HPO culture and the library culture were combined in a 2-L sterile flask. The mixture was incubated at 30℃, shaking 30 ~ 50 r/min for 24 h. The mating mixture was then spread on 50 SD/-Trp/-Leu/-His/-Ade agar plates (150 mm). Mating efficiency controls and positive control were set parallel. The plates were incubated, colony side down, at 30℃ for 16 ~ 21 days until colonies appear. Ade^+, His^+ colonies are white or light pink and can grow to >2 mm. These true colonies can grow on fresh SD/-Trp/-Leu/-His/-Ade/X-α-Gal agar plates and turn blue.

1.2.4 Analysis and verification of putative positive clones: Ade^+, His^+, $Mel1^+$ transformant colonies were selected for further analysis. Yeast plasmid DNA was isolated, purified and then transformed into *E. coli* strain DH5α according to the manufacturer's protocols. The AD/library inserts were amplified by PCR using the plasmid DNA isolated from *E. coli* as template. Primers were designed as below: Primer 1, 5′ CTA TTC GAT GAT GAA GAT ACC CCA CCA 3′ (forward primer); Primer 2, 5′ ACT TGC GGG GTT TTT CAG TAT CTA CGA 3′ (reverse primer).

The reaction mixture was denatured at 94℃ for 45 s, annealed at 55℃ for 45 s and extended at 72℃ for 60 s. Thirty cycles were performed and followed by a 10 min extension at 72℃. Fragments were digested by *Hae* Ⅲ and their sizes were analyzed by agarose gel electrophoresis. The interaction between candidate protein and HPO was verified by additional yeast two-hybrid interaction assay. Selected amplified DNA fragments were purified and sequenced by BIOASIA Co., Shanghai. The presence of an open reading frame (ORF) fused to the GAL4 AD sequence was verified and the sequences were compared to those in GenBank.

2 Results

2.1 Construction of bait plasmid pGBKT7-HPO

The inserting HPO gene fragment in pGBKT7 was verified by digesting and sequencing. pGBKT7-HPO was transformed into yeast strain AH109 and the expression of GAL4 DNA-BD/HPO was identified with anti-HPO antibody. Untransformed AH109 and pGBKT7 transformed AH109 were used as controls. Molecular mass of GAL4 DNA-BD /HPO fusion protein is about 33 ku. Figure 1 shows a single 33 ku band on lane 3 and no other band on the control lanes. The pGBKT7-HPO transformants were plated on SD/-Trp/X-α-Gal, SD/-Trp/-His/X-α-Gal, SD/-Trp/-Ade/X-α-Gal. Transformed AH109 with pGBKT7 was used as a control. AH109/pGBKT7-HPO colonies on SD/-Trp/X-α-Gal were white but could not grow on SD/-Trp/-His, SD/-Trp/-Ade. It can be concluded that DNA-BD/HPO protein could not activate reporter genes.

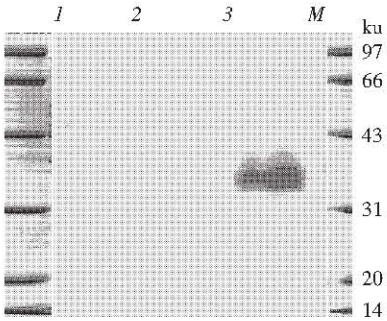

Fig. 1 Western blot analysis of expression of GAL4-BD fused HPO in AH109

1: AH109; *2*: AH109/pGBKT7; *3*: AH109/pGBKT7-HPO; *M*: Molecular mass marker. Protein extracts were fractionated on 12% SDS-PAGE. The Western blot was performed using anti-HPO polyclonal antibody.

2.2 Using HPO as bait to screen human testis library

HPO was expressed as a fusion with the GAL4 DNA-BD in AH109 (MATa). The high-complexity human testis cDNA library, which expresses fusions with the GAL4 AD, is provided in yeast strain Y187 (MATα). When the two transformant cultures are mated each other, diploid cells are formed which contain four reporter genes: *HIS*3, *ADE*2, *MEL*1 and *lacZ*. The GAL4 DNA-BD binds to the GAL UAS and, if the fusion proteins interact, the AD is brought into proximity to the reporter expression of α-(*MEL*1 product) and β-galactosidase (*lacZ* product). The true colonies could grow on SD/-Trp/-Leu/-His/-Ade/X-

α-Gal agar plates and turn blue. 69 robust blue colonies were selected for further identification.

2.3 Analysis and verification of putative positive clones

Plasmid DNA was isolated from candidate yeast colonies, purified and then transformed into *E. coli* DH5α. The plasmid DNA isolated from each positive yeast colony will be a mixture of the DNA/BD/HPO plasmid and at least one type of AD/library plasmid. Transformed *E. coli* cells were plated on LB medium containing ampicillin to select for transformants containing only the pACT2-AD/library plasmid. Amplify AD/library inserts by PCR using the plasmid DNA isolated from *E. coli* as template (Figure 2). Fragments were then digested by *Hae* Ⅲ and separated by agarose/EtBr gel electrophoresis. Fragment sizes were analyzed in order to eliminate colonies bearing the same AD/library plasmid. Twenty candidate colonies emerged from the previous 69 colonies.

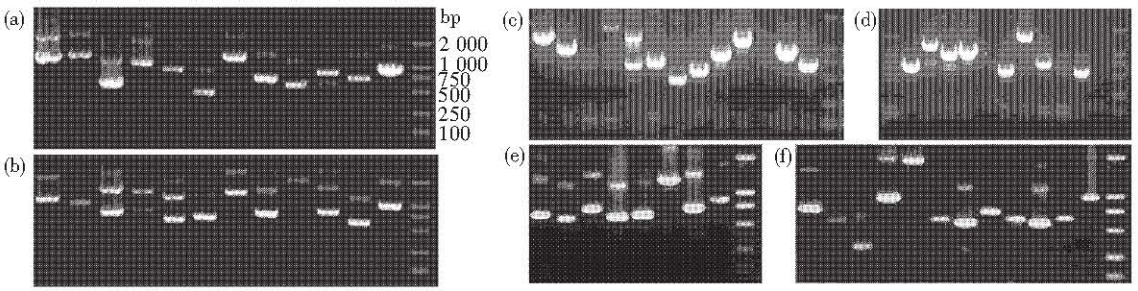

Fig. 2 Amplification of the pACT2-AD/library insert fragment in candidate yeast colony

1% agarose was used to separate the PCR product. DL-2000 was loaded as standard molecular mass marker and the different molecular mass were signed as in (a), same in (b, c, d, e) and (f).

The interaction between protein in candidate yeast colony and HPO was retested by additional yeast two-hybrid interaction assay. Selected amplified DNA fragments were purified and sequenced using the GAL4 AD sequencing primer (Clontech). The presence of an open reading frame (ORF) fused to the GAL4 AD sequence was verified and the sequences were compared to those in GenBank. The results revealed four HPO-interacting proteins: NADH dehydrogenase 1, ATP1β3, phospholipase C delta 1 and epididymal secretory protein. The Blast identities are 98% or above. Figure 3 offers partial cDNA sequence of the HPO-interacting proteins.

3 Discussion

Yeast two-hybrid system 3 provides immediate access to the genes encoding the interacting proteins. The assay is performed *in vivo* so that the proteins are more likely to be in their native conformations, which may lead to increased sensitivity and accuracy of detection. The four reporter genes designed in this system allow rapid identification of positive transformants.

We previously identified that extracellular HPO could trigger the MAPK pathway by binding its specific receptor and intracellular HPO specifically modulates the AP-1 pathway through JAB1[1~3]. We also demonstrate that HPO is a flavin-linked sulfhydryl oxidase[11]. But it is unclear why the expression level of HPO is so high in testis and which role HPO plays there. In this paper, we intend to find the clew about the biologic function of HPO in testis. HPO was used as bait protein to screen a human testis library by yeast two-hybrid assay. Our results offer four previously unknown HPO-interacting proteins.

NADH dehydrogenase 1 is an important component of complex Ⅰ (NADH: ubiquinone oxidoreductase), which is the first of electron transfer complexes participating in oxidative phosphorylation, and locates in the mitochondria intermembrane. 23 ku HPO is also located in mitochondria intermembrane space[5]. Na^+/K^+ ATPase uses the energy of one molecule of ATP to drive 3 sodium ions out of the cell and 2 potassium ions into the cell against substantial concentration gradients. The epididymal secretory protein is important for sperm maturation[12]. Phospholipase C is implicated in various signaling pathways and has been identified to be a regulator in fertilization[13]. Additionally, spermatozoa are enriched with mitochondria and need to expend large amount of energy during fertilization. The specific interaction between HPO and these proteins indicates that HPO may have close relationship of sperm development, fertilization, ATP synthesis and utilization, i. e. HPO may participate in cellular energy metabolism in testis/sperm and also regulate their biological functions. A consequence of these new data is the necessity to extend studies on HPO to different tissues, organs and developmental processes.

NADH dehydrogenase 1:

ATGATGAAGATACCCCACCAAACCCAAAAAAAGAGATCTCTATGGCTTACCCATACGATGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGGCCCCGGGG
ATCCGAATTCGCGGCCGCGTCGACAAAGAGCCCCTAAAACCCGCCACATCTACCATCACCCTCTACATCACCGCCCCGACCTTAGCTCTCACCATCGCTCTTCTACTATG
AACCCCCCTCCCCATACCCAACCCCCTGGTCAACCTCAACCTAGGCCTCCTATTTATTCTAGCCACCTCTAGCCTAGCCGTTTACTCAATCCTCTGATCAGGGTGAGCAT
CAAACTCAAACTACGCCCTGATCGGCGCACTGCGAGCAGTAGCCCAAACAATCTCATATGAAGTCACCCTAGCCATCATTCTACTATCAACATTACTAATAAGTGGCTCC
TTTAACCTCTCCACCCTTATCACAACACAAGAACACCTCTGATTACTCCTGCCATCATGACCCTTGGCCATAATATGATTTATCTCCACACTAGCAGAGACCAACCGAAC
CCCCTTCGACCTTGCCGAAGGGGAGTCCGAACTAGTCTCAGGCTTCAACATCGAATACGCCGCANGCCCCTTCGCCCTATTCTTCATAGCCGAATACACAAACATTATTA
TAATAAACACCCTCACCACTACAAATCTTTCCTAGGGAACAACATATTGACGCACTTCTTCCCTGGAAACTTTTACACAAACATAATTTTTGTCACCCAAGAACCCTTAC

Sodium/potassium-transporting ATPase beta-3 subunit:

CACGTGCTGTGATTTTGAACATAACTCGTCCCAAAAACTTGTCACGATCATCCTGACTTTTTAGGTTGGCTGATCCATCAGTCTTGCACTCAACTGTTACTTCTTTCCCA
GTGTTGTTAGGAGCAAAGCTGACCTGAACAGCAACCAATGGCTGTAGATACCCAACATGCAGTTTTTTCCCATAATATGGGAAATATTTTAAGTCTATCATTCCATTATG
AGGATAAACTGCTACATTTGGTATATCTTCATTCTTTGAAACACAATCTATCCTTGGCACTCCTTCAGGCTTTAATCCAATTATTCTGTTCATTTTCACAAGAATACAAG
GGTTTCCTTGAGAATAGCCAAAATCAGGATCATTCATACCACTGCATGCTTGAAGTAATGAAATAGGAAACTGACATGCAACATAAACTGGACCCTTCTGTTCAAAAAGT
GCTCCATCAGGACAGACTGTGAGGTTCTTCTGTTCTTCTAAAGTATATGGTTTTAGAAACTTCTTAAGGTCTTCAATGTACCCTGCATACGAAGTTGGATCAGACCTACT
GAATGTATATTCCAATGCGGTCACTGGTTTTGGAAAAACCATGAGTCCTGGGCTAGGAATCTGGTCACGGTATTTTGGAACCTCATCGTTGAGAGTCTGAAGCATAACCC
ACATCGTGAATGAGAAGAGTGCAAGCCAGGAACCCATAAAAAACTAGGTAGAAAGAAGCAAGATCAAACCCCAGCTCTTGGCGGNGCCGCCCCCAAGGAATTCGGGATCC
CCCGGGGCCTCCATGGGCCATA

Phospholipase C delta 1:

CTATGGCTTACCCATACGATGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGGCCCCGGGGATCCGAATTCGCGGCCGCGTCGACGACACGGAGTTTGCG
TTTGAGGTAGTTGTGCCTGACCTTGCCCTCATCCGCTTCTTGGTGGAAGATTATGATGCCTCCTCCAAGAATGACTTCATTGGCCAGAGTACCATCCCCTTGAACAGCCT
CAAGCAAGGATACCGCCATGTCCACCTCATGTCTAAGAACGGGGACCAGCATCCATCAGCCACCCTCTTTGTGAAGATCTCCCTCCAGGACTAGGCTGGAGGAAGCCAGT
GGGGTCCCCCCTGAGTGGGCTGGGCCCTCTGTCCACATGTGGGGACAGGGCTGGTGTGGCTGCTCCCAGCCTCTTGCTCAGAGCTAGGCCCCCAAATTGCCTTCAGCCCT
AACATAGTGTCTGCTGCTGCCTCCCTGGGGACCAGGAGCTAGCCCAGTCCCTGGAGCTGTCCTTCATTCCGTTAGGAATAACACTGCAGCCCTCTCCACCCTTCGGCCAG
CGAGTGGTCAAGGATTTTTATAAAAATCACGATTAGATTAAAAAA

Epididymal secretory protein:

CCCATACGATGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGGCCCCGGGGATCCGAATTCGCGGCCGCGTCGACCTGGCGCTCAGCACCGCTGCCCAGG
CCGAACCGGTGCAGTTCAAGGACTGCGGTTCTGTGGATGGAGTTATAAAGGAAGTGAATGTGAGCCCATGCCCCACCCAACCCTGCCAGCTGAGCAAAGGACAGTCTTAC
AGCGTCAATGTCACCTTCACCAGCAATATTCAGTCTAAAAGCAGCAAGGCCGTGGTGCATGGCATCCTGATGGGCGTCCCAGTTCCCTTTCCCATTCCTGAGCCTGATGG
TTGTAAGAGTGGAATTAACTGCCCTATCCAAAAAGACAAGACCTATAGCTACCTGAATAAACTACCAGTGAAAAGCGAATATCCCTCTATAAAACTGGTGGTGGAGTGGC
AACTTCAGGATGACAAAAACCAAAGTCTCTTCTGCTGGGAAATCCCAGTACAGATCGTTTCTCATCTCTAAGTGCCTCATTGAGTTCGGTGCATCTGGCCAATGAGTCTG
CTGAGACTCTTGACAGCACCTCCAGCTCTGCTGCTTCAACAACAGTGACTTGCTCTCCAATGGTATCCAGTGATTCGTTGAAAGAGGAGGTGCTCTGTAGCAAGAAACTG
AGCTTCCGGGTGGCTGGGTTNTCAANGGGTTGGCTCATGTCTCTTTTTCTGNCN

Fig. 3 Partial cDNA sequence of the candidate HPO-interacting proteins screened from human testis library

These cDNA inserts were amplified from the AD/library plasmid in *E. coli* cells and then sequenced (see above for details). *Eco*R Ⅰ site is underlined.

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肝细胞生成素在睾丸组织中的相互作用蛋白*

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摘要 肝细胞生成素（HPO）具有复杂的生理功能，在睾丸中的高表达提示其在生殖活动中的重要性，而不仅局限于肝再生. 构建了酵母表达载体 pGBKT7-HPO，采用酵母双杂交系统，以 HPO 为诱饵蛋白，从人睾丸 cDNA 文库中寻找能够与 HPO 相互作用的蛋白质. 经过筛选、验证阳性克隆，并进行 PCR、测序和序列比对，得到 4 种相互作用蛋白质：NADH 脱氢酶 1、钠/钾 ATP 酶 β3 亚基、磷脂酶 C δ1 以及附睾分泌蛋白. 提示 HPO 可能参与了细胞的蛋白质合成，能量代谢等. 通过对候选蛋白的研究，为探讨 HPO 对睾丸组织细胞功能的调节机制提供了重要的线索.

关键词 肝细胞生成素，酵母双杂交，蛋白质相互作用，人睾丸文库
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