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Short Communication

Interaction of Mouse Pem Protein and Cell Division Cycle 37 Homolog

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Abstract Mouse *Pem*, a homeobox gene, encodes a protein consisting of 210 amino acid residues. To study the function of mouse Pem protein, we used the yeast two-hybrid system to screen the library of 7-day mouse embryo with full-length mouse *Pem* cDNA. Fifty-two colonies were obtained after 1.57×10^8 colonies were screened by nutrition limitation and β-galactosidase assay. Seven individual insert fragments were obtained from the library, and three of them were identified, one of which was confirmed to be the cell division cycle 37 (Cdc37) homolog gene by sequencing. The interaction between mouse Pem and Cdc37 homolog was then confirmed by glutathione S-transferase pull-down assay, and the possible interaction model was suggested.

Key words *mPem*; cell division cycle 37 homolog; yeast two-hybrid system

The *Pem* gene was first obtained from the murine T-lymphoma library by differential screening [1]. The mouse *Pem* (*mPem*) gene is located on the X chromosome and encodes a protein consisting of 210 amino acid residues, which is distantly related to the *Drosophila* Prd/Pax family members, and resembles the homeodomain from residues 116 to 175 [2,3]. The *Pem* gene is expressed in a stage- and tissue-specific manner. *Pem* transcripts are abundant in 7–8-day mouse embryo, but decrease precipitously; on day 9, they become abundant in placenta and yolk sac until parturition [2]. Although *Pem* transcripts are not detectable in most tissues of the adult, they are present in the reproductive system, such as the testis, epididymis and ovary. *Pem* has also been shown to be specifically expressed in the proximal cauda and distal corpus region of the epididymis, the regions where spermatozoa gain forward motility and fertilization competence [4]. This implied an important role for *Pem* during embryogenesis and reproductive development.

In order to investigate the function of mPem, we used the GAL4-based yeast two-hybrid system to screen the 7-day mouse embryo library with the full-length *mPem* cDNA, trying to find proteins interacting with the Pem protein.

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Experimental Procedures

Matchmaker GAL4 two-hybrid system 3, including the yeast strain *Saccharomyces cerevisiae* AH109, pGBKT7, pGADT7 and the 7-day mouse embryo library, was purchased from Clontech (San Jose, USA). pEGFP-mPem plasmid containing the full-length cDNA of *mPem* was provided by Professor M. F. WILKINSON (Portland, USA). *Escherichia coli* DH5α was kept in our laboratory. The culture medium and amino acids were purchased from Amersco (Solon, USA). Reagents for molecular biology analysis were purchased from TaKaRa Bioengineering Company (Dalian, China) and Sigma-Aldrich (St. Louis, USA). [^{35}S]Met and glutathione-Sepharose beads were from Amersham (Piscataway, USA). TNT T7 quick coupled transcription/translation reaction kit was purchased from Promega (Madison, USA).

Primer 1 (5′-TTCCGTTCATATGGAAGCTGAGGGTTCCAGC-3′, forward primer, *Nde*I site underlined) and primer 2 (3′-CGCTGTGGCTCTAAAATTCAGCTGGGACAC-5′, reverse primer, *Sal*I site underlined) were designed for amplifying *mPem* cDNA from pEGFP-mPem. The full-length cDNA of *mPem* was cloned into pGBKT7 to construct pGBKT7-mPem, which was used as a bait to screen interacting proteins using the Matchmaker GAL4 two-

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hybrid system 3.

The pGBKT7-mPem and the 7-day mouse embryo Matchmaker two-hybrid library plasmids were co-transformed into *S. cerevisiae* AH109 competent cells using the LiAc method as described previously [5,6]. AH109 competent cell suspension (8 ml) was mixed with the pGBKT7-mPem (1 mg), the 7-day mouse embryo Matchmaker two-hybrid library plasmids (0.5 mg), Herring testes carrier DNA (20 mg) and 1×PEG3350/LiAc solution (60 ml), and incubated at 30 ºC for 30 min. After the addition of 7 ml dimethyl sulfoxide, the mixture was further incubated in a water bath at 42 ºC for 15 min. Finally the cells were centrifuged at 1000 *g*, resuspended in TE buffer (10 ml, pH 7.0), and incubated on SD/$^-$Trp$^-$Leu plates at 30 ºC for 48 h, then replicated onto SD/$^-$Trp$^-$Leu$^-$His and SD/$^-$Trp$^-$Leu$^-$His$^-$Ade plates in sequence.

β-galactosidase activity assay was performed as described previously [7]. Colonies were spotted onto Whatman #5 filter paper. The filter paper was then put into liquid nitrogen for 10 s, placed on the presoaked filter with 2 ml Z buffer (16.1 mg/ml $Na_2HPO_4{\cdot}7H_2O$, 5.5 mg/ml $NaH_2PO_4{\cdot}H_2O$, 0.75 mg/ml KCl, 0.25 mg/ml $MgSO_4{\cdot}7H_2O$), 5.4 μl purified β-mercaptoethanol, 33.4 μl X-Gal (20 mg/ml) and incubated at 30 ºC for 8 h. If blue appeared, it was a positive colony, and was selected for further analysis.

The plasmid DNA was isolated from the positive colony, purified and then transformed into *E. coli* DH5α cells using electroporation. The library inserts containing GAL4 AD were amplified by polymerase chain reaction (PCR) using the plasmid DNA isolated from *E. coli* as the template. The primers were designed as follows: primer 1, 5′-AAGTGAACTTGCGGGGTTTTTCAGTATCTA-3′; primer 2, 3′-CCAAACCACCCCATAGAAGTAGTAGCT-TAT-5′. The PCR conditions were: denatured at 94 ºC for 30 s, annealed at 56 ºC for 30 s and extended at 72 ºC for 60 s, for 30 cycles; followed by a 10 min extension at 72 ºC. Fragments were digested by *Hae*III and the sizes were analyzed by agarose gel electrophoresis. The selected clones were sequenced by Bioasia (Shanghai, China). The presence of open reading frames fused to the GAL4 AD sequences was verified and the sequences were compared with those in the GenBank.

The glutathione S-transferase (GST)-mPem plasmid was transformed into the *E. coli* strain BL21. The expression of the fusion protein was induced by the addition of 1 mM isopropyl-β-*D*-thiogalactoside and incubated at 37 ºC for 6 h. After lysing the bacterial cells by lysozyme, GST or the GST-mPem fusion protein were immobilized on glutathione-Sepharose beads as instructed by the manufacturer (Amersham). The beads were washed three times with binding/wash buffer (4.2 mM Na_2HPO_4, 2 mM KH_2PO_4, 140 mM NaCl, 10 mM KCl). Cdc37 labeled with [^{35}S]Met was expressed *in vitro* from the constructed plasmid pGBKT7-Cdc37 using a TNT T7 quick coupled transcription/translation reaction kit (Promega). Ten microlitres of the product was incubated with the beads for 1 h at room temperature. The beads were washed three times with binding/wash buffer and the bound proteins were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and scanned by Typhoon (Amersham).

Results

Using the LiAc method, the pGBKT7-mPem and the Matchmaker 7-day mouse embryo two-hybrid library plasmid were co-transformed into AH109 cells. We got 5.2×10^7 transformants from SD/$^-$Trp$^-$Leu plate. Plate 1.57×10^8 transformants on SD/$^-$Trp$^-$Leu$^-$His plates and then replicated on SD/$^-$Trp$^-$Leu$^-$His$^-$Ade plates. We obtained 298 colonies, of which 52 were confirmed to be positive colonies with β-galactosidase activity.

Plasmid DNA was isolated from the positive colonies, purified and then transformed into *E. coli* DH5α cells. The library inserts were amplified, digested by *Hae*III and separated by agarose/EtBr gel electrophoresis. Fragment sizes were analyzed to eliminate colonies bearing the same library plasmid. Seven candidate colonies from the 52 colonies confirmed to contain the single library plasmid were sequenced, then the sequences were compared with those in the GenBank. Three genes were identified and one of them was identical to the full-length coding region of *Cdc37*.

The *Cdc37* fragment inverted in pACT2-Cdc37 was amplified by PCR and constructed into pGBKT7 to form pGBKT7-Cdc37, and *mPem* was constructed into pGADT7 to result in pGADT7-mPem. The two recombinant plasmids were applied onto the yeast two-hybrid analysis to confirm their interaction. The yeast cells that were co-transformed with pGBKT7-Cdc37 and pGADT7 or pGADT7-mPem and pGBKT7 could not grow on SD/$^-$Trp$^-$Leu$^-$His$^-$Ade plates and showed no β-galactosidase activity. The yeast cells that were co-transformed with pGBKT7-Cdc37 and pGADT7-mPem could grow on SD/$^-$Trp$^-$Leu$^-$His$^-$Ade plates and showed strong β-galactosidase activity (**Fig. 1**), which indicated that mPem did interact with Cdc37 in yeast.

To substantiate the interaction between mPem and Cdc37, *in vitro* GST-pull down assay was performed. The Cdc37 labeled with [^{35}S]Met was expressed *in vitro* using the TNT T7 quick coupled transcription/translation reac-

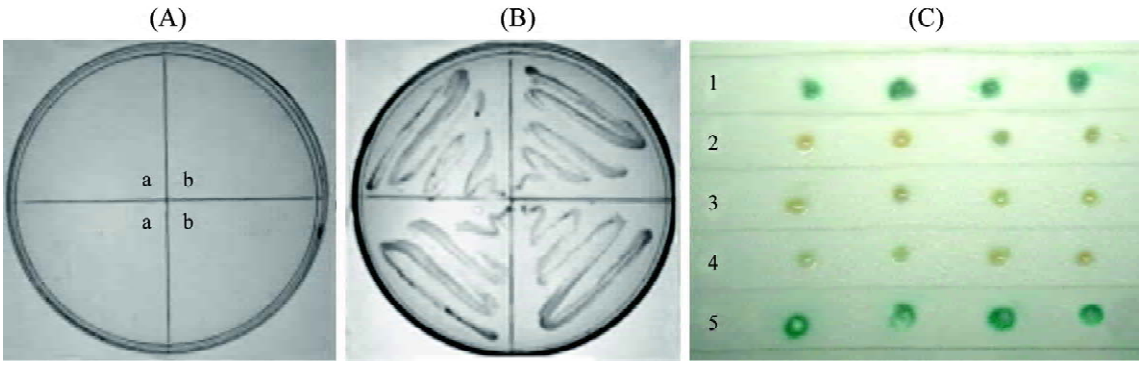

Fig. 1 Growth of transformants on SD/$^-$Trp$^-$Leu$^-$His$^-$Ade plates (A,B) and β-galactosidase activity assay of single transformants (C)

(A) SD/$^-$Trp$^-$Leu$^-$His$^-$Ade nutrition limitation test. a, pGBKT7-Cdc37/pGADT7 cotransformants; b, pGADT7-mPem/pGBKT7 co-transformants. (B) pGADT7-mPem/pGBKT7-Cdc37 co-transformants. (C) β-galactosidase assay. 1, positive control; 2, negative control; 3, pGBKT7-Cdc37/pGADT7 co-transformants; 4, pGADT7-mPem/pGBKT7 co-transformants; 5, pGADT7-mPem/pGBKT7-Cdc37 co-transformants.

tion kit. **Fig. 2** shows that Cdc37 can be pulled down by GST-mPem Sepharose beads, which indicates that Cdc37 can bind with mPem *in vitro*.

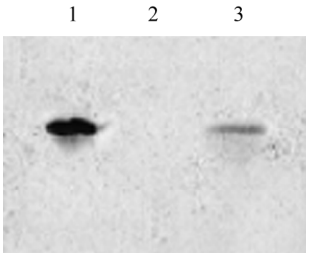

Fig. 2 Glutathione S-transferase (GST)-pull down assay of Cdc37 and mouse Pem proteins

In vitro expressed Cdc37 was incubated with immobilized beads and washed three times with binding/wash buffer. Bound proteins were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and scanned by Typhoon. 1, *in vitro* expressed products of [^{35}S]Met-labeled Cdc37; 2, GST Sepharose beads; 3, GST-mPem Sepharose beads.

Discussion

Specific protein-protein interactions are of fundamental importance for many biological processes. More and more studies have indicated that the Pem protein may play an important role in embryogenesis and reproductive development. Cdc37 was initially identified from *S. cerevisiae* as an essential factor for cell cycle progression through G_1 phase. It facilitates formation of Cdc28-G_1 cyclin complex [8], and helps many other kinases, such as Cak1, Stel1 and Mps1, in the stability and function of *S. cerevisiae* [9]. Cdc37 was originally identified as pp50 in mammalian cells as the component of protein complexes involving Hsp90 and oncogene protein pp60$^{v\text{-}src}$ [10], and later was found to be associated with various protein kinases, such as CDK11, Lck, heme-regulated elF2α kinase, Raf-1, MAK and MRK [11]. In most cases, Cdc37 is usually associated with Hsp90 in protein-kinase-client complexes and targets kinases to Hsp90 for activation. Biochemical and structural studies indicated that the N-terminal domain (residues 1–126) interacts with protein kinases, the middle domain (residues 128–282) interacts with Hsp90, and the C-terminal domain does not bind to Hsp90 or kinases and has no function [12]. In addition to the function of kinase activation, Cdc37 is critical for protein stability as the protein chaperone in the Hsp90-independent model [13]. Some Hsp90/Cdc37-dependent client proteins are not kinases, for example, the androgen receptor and hepadnavirus reverse transcriptase [14,15].

Because of its specific expression in the reproductive system, such as the testis, epididymis and ovary, Pem was believed to play an important role during embryogenesis and reproductive development. Studies on the *Pem* gene showed it had two promoters: one was a distal promoter (Pd) which was active in placenta, ovary, tumor cell line and, to a lesser extent, in skeletal muscle; the other was a proximal promoter (Pp) which was active in testis and epididymis [3,16]. This implicated the time- and tissue-specific expression of the *Pem* gene might be regulated by its promoters.

Cdc37 has significant effects on cell proliferation due

to its downregulation or upregulation. In fission yeast, switching off the expression of Cdc37 resulted in cessation of both growth and cell division [8,11]. Cdc37 upregulation is a common early event in localized human prostate cancer. Cdc37 overexpression drives proliferation, but loss of Cdc37 arrests growth and leads to apoptosis. Molecular analysis of Cdc37 client pathways demonstrated that the increased expression of Cdc37 resulted in enhanced Raf-1 activity, increased Cdk4 level, and reduced expression of cyclin-dependent kinase inhibitor [17]. A lot of data indicated that Cdc37 induces proliferation and was critical for the survival of human prostate epithelial cells by regulating the kinases in the MAPK signal pathway.

It is well known that interaction with other proteins is crucial for a homeobox-containing protein to exert its function [18]. The interaction of mPem with Cdc37 helps uncover its function in reproduction. mPem may regulate the development of the reproductive system in mPem/Cdc37 or mPem/Cdc37/Hsp90 through the MAPK signal pathway. However, this hypothesis needs to be verified in the future.

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