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Genetic Screening of the Dazl-Interacting Protein Genes

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Abstract Micro-deletions at specific loci of the Y chromosome have been observed frequently in male infertility patients, suggesting that genes in these regions are involved in male germ cell development. *DAZ* is a representative male infertility gene at the *AZFc* locus of the Y chromosome. Since DAZ contains an RNA binding motif along with so-called a DAZ domain, it was proposed to participate in RNA metabolism during spermatogenesis. A mouse gene homologous to the human *DAZ* gene has been cloned and named *Dazl* (*DAZ-like*). *Dazl* is autosomal and expressed in the testis and also at a low level in the ovary. Male mice homozygous for the *Dazl* null allele have small testes with a few spermatogonia and almost complete absence of germ cells beyond the spermatogonial stage, suggesting the requirement of *Dazl* for entry or progression through meiosis. However, its exact cellular functions have not been understood yet. In order to investigate cellular functions of *Dazl*, we decided to isolate candidate interacting protein genes of the mouse *Dazl*, using yeast two-hybrid screening. A number of candidate Dazl-interacting proteins have been isolated, such as Bprp, Acf, Hgs, Murr1, Nbak3 and Ranbp9, but dynein light chain 1 (Dlc1) was most predominant. A strong interaction of Dazl with Dlc1 suggests that Dazl might function as an mRNA adaptor to the dynein motor complex.

Key words: DAZ, Dazl, male infertility, yeast two-hybrid screening, Dlc1

INTRODUCTION

The Y chromosome in mammals dictates the formation of the testis. Many of the Y chromosome genes show testis-specific expression and are involved in spermatogenesis (Vogt et al., 1996; Lahn and Page, 1997). In accordance, a high percentage of infertile men have deletions in the Y chromosome sequences (Roberts, 1998; Cooke, 1999; McElreavey and Krausz, 1999). *DAZ* (*Deleted in AZoospermia*) is one of the genes located at the *AZFc* region of the Y chromosome. Since micro-deletions at the *AZFc* locus have been frequently observed in the azoospermic and oligospermic males, *DAZ* was considered a candidate male infertility gene (Reijo et al., 1995, 1996a).

There are three *DAZ* family genes in the human genome: *DAZ*, *DAZL* and *BOULE*. *DAZ* is detected only in the Y chromosomes of great apes and Old World monkeys, yet all mammals contain autosomal *DAZL* and *BOULE* (Shan et al., 1996; Cooke et al., 1996; Reijo et al., 1996b; Saxena et al., 1996; Yen et al., 1996; Seboun et al., 1997). It was proposed that the *DAZ* family genes were originated from duplication-transposition of an ancestral autosomal *BOULE*, followed by amplification and pruning (Saxena et al., 1996). The genomic structures of *DAZ* and *DAZL* share a high degree of homology not only along the coding sequence but also in the promoter region and introns (Saxena et al., 1996; Chai et al., 1997). Both the genes encode RNA-binding proteins that are expressed exclusively in germ cells.

Mouse genetic studies revealed that *Dazl* is also critical for male germ cell development. Male mice homozygous for the *Dazl* null allele have small testes with a few spermatogonia and almost complete absence of germ cells beyond the spermatogonial stage, suggesting the requirement of Dazl for entry or progression through meiosis (Ruggiu et al., 1997; Saunders et al., 2003). Introduction of the human *DAZ* gene into *Dazl*$^{-/-}$ mice resulted a partial and variable rescue of the mutant phenotype, suggesting that the high degree of functional conservation between the human *DAZ* and mouse *Dazl* genes (Slee et al., 1999). However, exact cellular functions of the Dazl protein are still remained to be investigated.

To shed light on how Dazl functions in mouse germ cells, we carried out yeast two-hybrid screening with Dazl as a bait. The results showed that the *dynein light chain 1* (*Dlc1*) gene is the most predominant species of the Dazl-interacting protein genes.

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MATERIALS AND METHODS

Plasmid constructs

The mouse *Dazl* cDNA was PCR-amplified from the mouse testis RNA and subcloned into the *pGEM-T* Easy vector (Promega). For yeast two-hybrid screening, the full open reading frame (ORF) of *Dazl* was subcloned into the *EcoR*I site of *pGBT9* (containing the *GAL4* DNA-binding domain) and *pLexA* (containing the *LexA* DNA-binding domain). Four *Dazl* truncated mutants ($Dazl^{1\text{-}166}$, $Dazl^{1\text{-}190}$, $Dazl^{115\text{-}298}$, and $Dazl^{39\text{-}298}$) were generated and subcloned into the *EcoR*I site of *pGBT9*.

Yeast two-hybrid screening

Two different yeast two-hybrid systems were used for this study: The *GAL4*- and *LexA*-based systems. For the *GAL4*-based system, the MATCHMAKER Two-Hybrid System (Clontech) was used. The *pGBT9/Dazl* plasmid was co-transformed with a mouse testis cDNA library into a yeast reporter strain, *Saccharomyces cerevisiae CG-1945*. The Dazl expression in the yeast cells were confirmed by the immunoblot analysis with the polyclonal antibodies specific to Dazl (Lee et al., 2006). A mouse testis MATCHMAKER cDNA library in the *pACT2* vector was purchased from Clontech. Screening of the library for interacting proteins was carried out according to the manufacturer's manual. Approximately 5×10^6 colonies were screened with a co-transformation efficiency of about 5×10^3 cfu/mg of the mouse testis library DNA. The transformants were plated on the SD/-His/-Leu/-Trp medium with 5 mM 3-amino-1,2,4-triazole (3-AT) and incubated at 30°C for 3-6 days. The specificity of the yeast two-hybrid interaction was confirmed further with the β-galactosidase assay. Expression of β-galactosidase in the yeast colonies was determined using a colony-lift filter assay. Plasmid DNA from positive clones was transformed into the *E. coli MH4* strain grown on the M9 agar plates (Sambrook et al., 1989). The cloned genes were identified by sequencing or by restriction enzyme digestion.

For the *LexA*-based system, the *pLexA/Dazl* plasmid was transformed into the *Saccharomyces cerevisiae EGY48 [p80p-lacZ]Ura3* strain (Invitrogen). Afterward, the HeLa cDNA library in the *pJG4-5* vector (Invitrogen) was transformed sequentially. Approximately 2×10^7 colonies were screened with a sequential transformation efficiency of about 2×10^4 cfu/mg. The transformants were plated on the SD/Gal/Raf/-His/-Leu/-Trp/-Ura medium with 5 mM 3-AT and incubated at 30°C for 3-6 days. The positive colonies were subjected to the b-galactosidase assay. The plasmid DNA from the positive clones was transformed into *E. coli KC8* strain and grown on the M9 agar plates (Sambrook et al., 1989). The cloned genes were identified by sequencing or by restriction enzyme digestion.

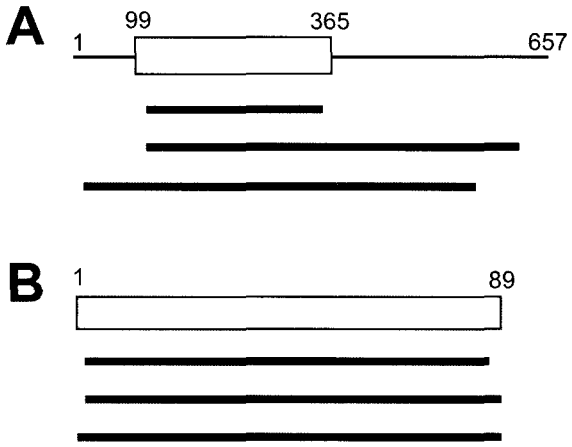

Fig. 1. The *Dlc1* clones isolated from yeast two-hybrid screening with Dazl as a bait. A, The full-length cDNA clone of *Dlc1* is shown. The ORF of *Dlc1* cDNA is depicted as a blank box. The numbers indicate positions of the *Dlc1* nucleotide sequence. The bold lines indicate three representative clones of *Dlc1* that were obtained from yeast two-hybrid screening with Dazl as a bait. B, The structure of Dlc1 protein is shown as a blank box. The numbers indicate positions of the Dlc1 amino acid residues. The bold lines indicate the Dlc1 protein sequences that are encoded in the isolated clones.

RESULTS

In order to have clues on biological functions of the mouse Dazl protein during the male germ cell development, we carried out yeast two-hybrid screening with Dazl as bait. Screening the mouse testis library using a *GAL4*-binding domain system, we fished out the *Dlc1* gene. To our surprise, all the isolated positive clones in the screen turned out to be *Dlc1*. Concerning artificiality in the screening system, we decided to adopt another yeast two-hybrid screening system. When the HeLa cDNA library was screened in a *LexA*-based system, *Dlc1* was again the only gene that was fished out. Most of the isolated Dlc1 clones contained in-frame full ORF sequences, indicating that Dlc1 is indeed a candidate Dazl-interacting protein (Fig. 1).

Since two independent screenings resulted in *Dlc1* as the only Dazl-interacting protein gene, we decided to screen the mouse testis library with Dazl-truncated mutants as baits, hoping to isolate additional Dazl-interacting protein genes (Fig. 2A). The results showed that *Dlc1* was still the most predominant species with the Dazl-truncated mutants (Fig. 2B). In addition, we were able to detect several additional clones other than *Dlc1* (Table 1). Among them, *Dazap2/Bprp* clones were detected four times while the others were detected once. *Dazap2* was named as a candidate of DAZ interacting protein previously (Tsui et al., 2000). *Dazap2* encodes a protein of 167 amino acids in size and proline-rich, but lacks a recognizable functional motif and a significant homology to other proteins. The isolated *Dazap2/Bprp* clones contained different parts of the ORF sequence but

Table 1. Clones identified from yeast two-hybrid screen with Dazl as a bait

Identity	No. of clones	Description	Reference
Dlc1	>20	Dynein light chain 1	Strausberg *et al.* (2002)
Bprp/Dazap2	4	Proline-rich protein expressed in brain, DAZ-associated protein 2	Tsui *et al.* (2000)
Arf	1	*Mus musculus* similar to APOBEC-1 complementation factor	XM_132112
Hgs	1	HGF-regulated tyrosine kinase substrate	Komada *et al.* (1995)
Nurr1	1	*Mus musculus* U2af1-rs1 region1	Nabetani *et al.* (1997)
Nbak3	1	Nuclear body associated kinase 3	Kim *et al.* (1998)
Ranbp9	1	RAN binding protein 9	Nakamura *et al.* (1998)

are in-frame, suggesting that it is a candidate Dazl-interacting protein (Fig. 3). In addition, we isolated 5 additional clones as listed in Table 1.

Acf (APOBEC1 complementation factor or APOBEC1-stimulating protein) encodes an APOBEC-1 stimulating protein and is known to play an important role in deamination of cytidine (Mehta et al., 2000). APOBEC1 transports ACF as cargo to and from the nucleus. ACF binds to the RNA substrate, facilitates the binding of APOBEC1 to the RNA. *Hgs (Hrs)* encodes a HGF-regulated tyrosine kinase substrate (Komada and Kitamura, 1995). Hgs is a 115-kDa double zinc finger protein that is rapidly tyrosine phosphorylated in growth factor-stimulated cells. Hgs may be involved in protein traffic through early endosomes (Komada et al., 1997). *Murr1* encodes an U2af1-rs1 region protein (Nabetani et al., 1997). The mouse *Murr1* gene contains an imprinted gene, *U2af1-rs1*, whose functions remain to be investigated. *Nbak3* encodes a nuclear body associated kinase 3 (Kim et al., 1998). Nbak3 is a functionally active Ser/Thr kinase, and it appears to be the first putative steroid receptor regulatory protein. *Ranbp9* encodes a Ran binding protein 9 (Nakamura et al., 1998). Ranbp9 serves as receptor for nuclear localization signals (NLS) in cargo substrates and mediates docking of the importin/substrate complex to the nuclear pore complex (NPC) through binding to nucleoporin. Rabbp9 mediates the nuclear import of RPS7, RPL18A and H2B histone and prevents the cytoplasmic aggregation of RPS7 and RPL18A by shielding exposed basic domains. It may also import RPL4, RPL6 H2A, H3 and H4 histones (Muhlhausser et al., 2001).

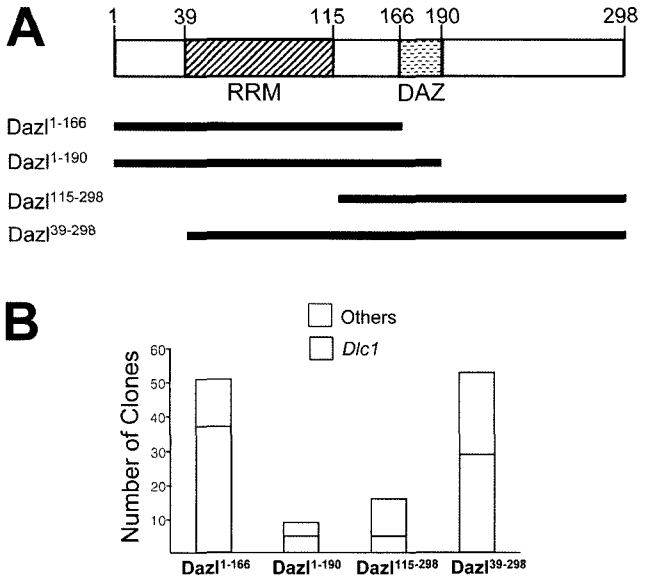

Fig. 2. The number of *Dlc1* clones obtained from yeast two-hybrid screen with the Dazl truncated mutants as baits. A, Schematic drawing of the truncated *Dazl* mutant constructs used as baits for yeast two-hybrid screening. B, Number of clones obtained with indicated *Dazl* mutant constructs. The gray boxes indicate the number of *Dlc1* clones.

DISCUSSION

In the present study, we identified Dlc1 as a candidate Dazl-interacting protein gene. Dlc1 is the smallest component of

Fig. 3. The *Bprp/Dazap2* clones isolated from yeast two-hybrid screening with Dazl mutants as baits. A, The full-length cDNA clone of *Bprp/Dazap2* is shown. The ORF of *Bprp/Dazap2* cDNA is depicted as a blank box. The numbers indicate positions of the *Bprp/Dazap2* nucleotide sequence. The bold line indicates a clone of *Bprp/Dazap2* that were obtained from yeast two-hybrid screening with Dazl as a bait. B, The structure of Bprp/Dazap2 protein is shown as a blank box. The numbers indicate positions of the Bprp/Dazap2 amino acid residues. The bold lines indicate the Bprp/Dazap2 protein sequences that are encoded in the isolated clones.

the cytoplasmic dynein motor complex. In lower eukaryotes, the axonemal dyneins are necessary for the motility of cilia and flagella (King and Patel-King, 1995). In higher eukaryotes, microtubule-associated cytoplasmic dyneins have been implicated in a number of cellular functions including cytoplasmic organelle movement, retrograde transport in axons, nuclear migration as well as in positioning and possibly assembly of the mitotic spindle (Vallee and Sheetz, 1996). Since our results suggested that Dazl might interact with Dlc1, it is possible that Dazl is associated with the dynein motor complex and involved in subcellular transport mechanisms.

Since Dazl contains RNA binding motif, it is possible that Dazl plays as an adaptor of specific RNAs to the dynein motor complex. In fact, association of RNA-binding proteins with the dynein motor complex has been reported in *Drosophila*. The *bicoid* mRNA is transported and anchored to the anterior end of the *Drosophila* oocytes. Dynein is responsible for *bicoid* transport, and a number of additional proteins such as Exuperantia, Swallow, g-tubulin37C, Grip75, and Staufen are also involved in the transport and retention of *bicoid* at the anterior end of the oocytes throughout oogenesis (Ferrandon et al., 1994; Schnorrer et al., 2000; Cha et al., 2001; Schnorrer et al., 2002). In particular, Swallow was known to interact with the Dlc, suggesting its function as an adaptor for *bicoid* mRNA to dynein (Schnorrer et al., 2000).

Previously, Tsui et al. (2000) carried out the yeast two-hybrid screening with human DAZ as bait and isolated two novel clones named *Dazap1* and *Dazap2/Bprp*. Another yeast two-hybrid screening by Moor et al. (2003) reported *Pumilio-2* as a candidate DAZ-intereacting protein gene. However, neither groups reported *Dlc1* as a DAZ-interacting protein gene. This is extraordinary since *Dlc1* was the most predominant species for us. One possibility may be that Dlc1 interacts with Dazl but not with DAZ. Although Dazl and DAZ share a high structural homology, they may interact with different sets of proteins for distinct biological functions. Nevertheless, some of their functions may be redundant, as shown that Dazap2 interacts with both Dazl and DAZ.

Since we identified Dlc1 as a most predominant interaction with Dazl, we will focus on elucidating interaction of Dazl with Dlc1 in the future.

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