Identification and characterization of rDJL, a novel member of the DnaJ protein family, in rat testis

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Abstract Applying the method of segmentation of seminiferous tubules combined with DDRT-PCR and cDNA library screening, a novel DnaJ homologue, rDJL, was identified in rat testis. The reading frame encodes a protein of 223 amino acid residues containing J domain in the NH2 terminal region. rDJL gene is expressed mainly in testis and rDJL protein was immunolocalized notably in the acrosome region of spermatozoa. Immunoprecipitation experiments showed that rDJL interacted with Hsc70 and clathrin protein. When CHO cells were treated with EGF, rDJL and clathrin protein were found to be colocalized and be concentrated as endosome vesicles. The present findings suggest that rDJL functions as co-chaperone to Hsc70, participates in vesicular trafficking and may play an important role in acrosomogenesis.

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1. Introduction

Mammalian spermatogenesis involves an intricate progression of cell division and differentiation leading to the formation of mature spermatozoa, whereby some crucial genes are expressed under stringent temporal and spatial regulation. Identification and characterization of these differentially expressed genes will be of great value in delineating the mechanism of spermatogenesis. In the present study, the method of differential display polymerase chain reaction (DDRT-PCR) combined with segmentation of rat seminiferous tubules was applied to investigate the differential gene expression in germ cells at different stages of spermatogenesis [1]. One of the expressed sequence tags (ESTs) was isolated and used as probe in screening a cDNA library of rat testis, whereby a full-length cDNA (GenBank Accession No. AF154849) was identified.

The encoded protein contains the conserved J domain in the N-terminal region thus was designated as rDJL—a novel member of the DnaJ protein family. It is well established that DnaJ proteins serve as co-chaperones to Hsp70 proteins [2] and are required for the stimulation of interacting Hsp70 ATPase activity [3,4]. DnaJ proteins are involved in a variety of processes such as protein folding [5], protein trafficking [6,7], signal transduction [8], regulation of gene expression [9], uncoating of clathrin-coated vesicles [10], and so on. To date several DnaJ proteins involved with spermatogenesis have been identified. MSJ-1 is a DnaJ homolog specifically expressed in germ cells during the haploid stages, and interacts with mUBPy. It may be important for acrosome formation and centrosome adjustment during spermatid development [11,12]. Studies on the role of DjA1, a type I DnaJ homolog, revealed that deletion of this gene in mice led to severe defects in spermatogenesis, involving aberrant androgen signaling [13].

To characterize rDJL, the transcripts of this gene in multiple tissues and testis in developing at varying postnatal days were determined by quantitative real-time PCR and Northern blot assay. The highest level of rDJL transcripts were found in rat testis, and the earliest evidence of expression occurred in testis on the 30th postnatal day, which reached adult level on the 60th postnatal day. Immunofluorescent studies revealed that the protein was localized in the acrosomal region of spermatozoa. Immunoprecipitation experiments confirmed that rDJL interacts with Hsc70 and clathrin protein. Evidence will be presented showing that rDJL functions as co-chaperone to Hsc70 and participates in vesicular trafficking, thus may be important for acrosome formation during spermiogenesis.

2. Materials and methods

2.1. Differential display of mRNA and screening of the rat testis cDNA library

The cycle of rat seminiferous epithelium can be divided into 14 stages [14,15] and each stage represents a different combination of germ cell status, including spermatogonia, spermatocytes, spermatids and spermatozoa. Based on the morphological features of these germ cells, the stages in the cycle of the seminiferous epithelium can be delineated by transillumination and seminiferous tubule divided into four segments of stages II–VI, VII–VIII, IX–XII and XIII–I. In this research, two segments of rat seminiferous tubule in stages IX–XII and XIII–I were isolated under a dissecting microscope, delineated by the use of differential diopeters.
Total RNA was prepared and differential display was performed as described by Liang et al. [16] using 200 μg total RNA from each preparation. cDNA (1 μl) was added to the PCR mixture containing [α-35S] deoxyadenosine triphosphate (10 mCi/ml; Amersham), 10-mer deoxyoligonucleotide random 5′ primer, 3′-oligo dT14 (N is A, C, or G) single-anchored primer. The radiolabeled DNA fragments were electrophoresed in 6% denaturing polyacrylamide gels, and after drying, the gels were exposed to X-ray films. Differentially expressed cDNAs were cut from the dried gels and incubated in 50 μl dH2O and the DNA extracted by boiling.

The isolated cDNAs were reamplified and the products denatured and dotted onto two identical nylon membranes with equal amounts and in the same array pattern. The membranes were hybridized with radiolabeled cDNA probes prepared with total RNAs from the two segments of seminiferous tubule, respectively. Hybridization was performed at 65°C for 30 h in hybridization solution (1 mM EDTA, 7% sodium dodecyl sulfate, 0.5 M Na2HPO4, pH 7.2). The membranes were washed twice with 2× sodium saline citrate at room temperature, each for 15 min, then washed at 37°C for 30 min and at 65°C for 30 min. Autoradiography was performed at ~70°C. Those cDNA fragments, whose expression differences had been confirmed by reverse dot-blot hybridization were cloned into pUC19 plasmid and sequenced and submitted to GenBank as ESTs.

One of the ESTs derived from differential displayed mRNA of fragmenting seminiferous tubule (GenBank Accession No. AF059656) was used as probe in screening a rat testis gt10 5′-stretch cDNA library (Clontech, CA). A total of 1×106 clones were screened with the plaque hybridization method. Positive clones were selected and the inserts amplified, using the primers of the flanks sequence of phage vectors. The amplified products were cloned into pGEM-T Easy vector (Promega), sequenced using the ABI 377 autosequencer. The sequence was deposited with GenBank.

2.2. Northern blot and real-time PCR assay

Tissues of brain, heart, intestine, kidney, liver, lung, muscle, spleen and testis were dissected from adult male rats, frozen in liquid nitrogen immediately, and stored at ~80°C. In the developmental studies, testes were collected from male rats at days 7, 10, 20, 30, 45, 60, 80 and 120 of neonatal life. Total RNAs were prepared, using the Trizol reagent (Invitrogen).

In the Northern blot analysis, 20 μg of each sample was subjected to electrophoresis on a 1% agarose/formaldehyde gel and transferred onto a positively charged nylon membrane (Boehringer Mannheim). Radiolabeled cDNA probes were prepared using [α-32P] deoxyctydine triphosphate. Autoradiography was performed to visualize the comonomer showing positive hybridization with the probe. Blots were stripped and reprobed for β-actin.

Quantitative real-time PCR analysis was performed on 2 μg of total RNA from each species of rat tissue which was used for the first-strand cDNA synthesis utilizing the SuperScript First-strand Synthesis System (Invitrogen). The real-time PCR reaction was performed in a volume of 20 μl containing oligonucleotide primers (5 μM each), and SYBR Green PCR Master Mix (Applied Biosystems) containing Taq DNA Polymerase, the reaction buffer, dNTP and the double strand DNA-specific fluorescent dye SYBR Green. Amplification was performed as a two-step procedure: denaturation at 95°C for 10 min and 40 cycles with denaturation at 95°C for 15 s, annealing and elongation at 60°C for 1 min. The fluorescent signal from the samples was measured at the end of the elongation step. The sequences of the primers for rDJL were: forward; 5′-TTT CCA TCA ATT TCC AAC CAC G-3′, and reverse; 5′-ACT CTG AAC CCT TTT TGG CTT-3′; The sequences of the primers for β-actin were: forward; 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′, and reverse; 5′-TAA GCA GCT CAG TAA CAG TCC G-3′.

2.3. Preparation, purification of recombinant protein and raising of anti-rDJL antisemur

An rDJL cDNA fragment, encoding essentially the C-terminal portion of rDJL protein (aa 82–223) was cloned in frame with glutathione S-transferase (GST) using the pGEX-4T-3 vector. The recombinant GST-rDJL(82–223) protein was expressed in Escherichia coli BL21 (DE3) and purified by chromatography on Glutathione Sepharose 4B (Amersham Biosciences) according to standard procedures. The purity of the fusion proteins was judged by SDS-PAGE and Coomassie blue-staining. The protein concentration was assessed by comparison with BSA standards using BCA™ Protein Assay (Pierce). New Zealand rabbits were immunized with GST-rDJL(82–223) protein to raise polyclonal antibodies. Antibody titer of the anti-rDJL antiserum was determined by enzyme-linked immunosorbent assay (ELISA). To establish the specificity of the antiserum, Western blot assay was carried out by testing purified protein samples of GST-rDJL(82–223) and rat testis extract with the antiserum and preimmunized serum.

2.4. Immunohistochemical localization

Cryostat rat testis sections of 5 μm-thick were prepared and processed according to the manufacturer’s instruction (Zymed Histostain-SP, MT Rabbit ACE kit, Zymed Lab. Inc. South San Francisco). After blocking with 5% BSA in 3% H2O2/PBS buffer, the testis sections were incubated with anti-rDJL antibody (1:1000 dilution in PBS/5% BSA), or with preimmune serum (1:1000 dilution in PBS/5% BSA) as negative control for 1 h at room temperature. After washing three times with PBS buffer, all slides were incubated with biotin-labeled anti-rabbit IgG antibody at 37°C for 30 min and then incubated with Streptavidin-coupled horseradish peroxidase at 37°C for 30 min. Sections were washed with PBS, incubated with AEC detection buffer (4 mg AEC dissolved in 1 ml dimethyl formamide and 14 ml of 0.1 M sodium acetate, pH 5.2, with 15 μl of H2O2), and counterstained with Mayer’s haematoxylin and examined by light microscopy.

2.5. Cyto-immunofluorescent localization

The separation of germ cells was performed as previously reported [17]. Isolated spermatogenic cells were smeared onto microscope slides and fixed with paraformaldehyde fixative. The slides were washed three times in PBS and the cells were permeabilized by treatment with 0.5% Triton in PBS for 10 min. The slides were washed three times with PBS and blocked by incubating in PBS containing 3% bovine serum albumin for 15 min. For the detection of rDJL, the cells were incubated first with rabbit antiserum against rDJL, then incubated with FITC-labeled goat antibody against rabbit IgG and finally counterstained with Hoechst 33258. The slides were examined under confocal microscope.

2.6. Co-immunoprecipitation assay

The coding region of rDJL was subcloned into pCDNA6/V-HisB-HA vector (HA tag sequence inserted into pCDNA6/V-HisB vector between Nhel and HindIII sites). HEK293 cells were transiently transfected with pCDNA6/V-HisB-HA-rDJL, and pEGFP-C3-clathrin expression vectors (kindly provided by Dr. Lois E. Greene, National Institutes of Health, Bethesda, MD) [18]. Lipofect AMINE™ was used according to the manufacturer’s instructions (Invitrogen, CA). Cells were lysed in EBC (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 0.5% NP40, 50 mM NaF, 50 μg/ml PMSF, 10 μg/ml aprotinin and leupeptin). Whole cell extracts were incubated with anti-HA monoclonal antibody (Santa Cruz Biotechnology). Protein A-agarose beads (40 μl) were added and the mixture incubated and rotated overnight at 4°C. The beads were washed with NETN (20 mM Tris–Cl, pH 8.0, 100 mM NaCl, 0.5% NP40, and 1 mM EDTA). The proteins were collected, resuspended in SDS-PAGE sample buffer, and subjected to SDS-PAGE and Western blot using anti-HC70 antibody or anti-GFP antibody (Santa Cruz Biotechnology).

2.7. Localization examination of rDJL and clathrin protein in CHO cells

The coding region of rDJL was subcloned into pDrRe1-N1 vector. CHO cells were transiently transfected with pDrRe1-N1-rDJL and pEGFP-C3-clathrin and grown on coverslips. Thirty-six hours after post-transfection, cells were treated with 100 nM epidermal growth factor (CytoLab) for 15 min at 37°C. After stimulation, the cells were fixed with 4% formaldehyde and then visualized by confocal microscopy.

3. Results

3.1. Isolation and identification of rDJL gene

The full-length cDNA of rDJL was isolated by screening a rat testis cDNA library, sequenced and was assigned the
GenBank Accession No. AF154849. It consisted of 950 bp, containing an open reading frame of 669 bp with 5′ and 3′ noncoding regions of 130 and 148 bp, respectively (Fig. 1). The deduced protein contained 223 amino acid residues and was found to be homologous to members of the DnaJ family. It contained the canonical J domain (residues 3–69) including the conserved tripeptide, His-Pro-Asp, and the 50th Ala residue. It however, lacked the Gly/Phe-rich or the Cys-rich regions which occurred in many DnaJ-like proteins, so belongs to the type III subgroup of DnaJ proteins.

RNAs were isolated from various rat tissues and analyzed to determine the tissue expression pattern of rDJL transcripts. The result of real-time PCR showed that the rDJL transcripts were most abundant in testis, followed by liver; whereas other tissues contained inconsequential amounts (Fig. 2). To determine whether rDJL expression changes with development, total RNAs were prepared from testes of rats at different postnatal ages and analyzed by Northern blot. As shown in Fig. 3, rDJL was initially detected in the testis of day 30 postnatal rat and its expression level reached adult level on the 60th postnatal day. These findings suggest that the expression of rDJL gene is developmentally regulated and occurred predominantly during the haploid stages of spermatogenesis.

3.2. Identification of rDJL protein by rDJL antiserum

To obtain an antiserum specifically against rDJL, we produced a recombinant fusion protein, GST-rDJL(82–223), which
contains the carboxyl-terminal portion of rDJL but not the J domain. The expressed fusion protein was purified by Glutathione Sepharose 4B column chromatography (Fig. 4).

Using the purified recombinant protein as antigen, antiserum with high titer was raised in rabbits (ELISA data not shown). Western blot analysis showed that the antiserum reacted with the purified recombinant protein, and with a specific component found in the spermatogenic cell lysate (Fig. 5). The positive stained band corresponded to a protein of about 30 kDa, which is in agreement with the predicted molecular mass of rDJL protein.

The localization of rDJL protein in germ cells of normal rat testis at various stages of differentiation was determined by immunohistochemical technique (Fig. 6). Meiotic spermatocytes, round spermatids, elongating spermatids and testicular spermatozoa were immunopositive; whereas spermatogonia resting directly on the basal lamina were immunonegative. It is noteworthy that the testicular interstitial tissue cells were not stained, demonstrating that rDJL is a gene product restricted to germ cells of the seminiferous epithelium.

To localize more precisely the subcellular sites of rDJL, isolated spermatogenic cell suspensions were freshly prepared, fixed and processed for immunofluorescence microscopy. Samples were counterstained with the DNA intercalating dye Hoechst 33258. In spermatocytes, rDJL showed a diffuse distribution in the cytoplasm. In spermatids, rDJL had a remarkable asymmetrical perinuclear localization that is coincident with that of the developing acrosome. Spermatozoa showed a well delineated immunostained acrosome and, in addition, exhibited staining of the tail (Fig. 7). The result of immunofluorescence microscopy suggested that rDJL may play a role in the biogenesis of the acrosome.

3.3. rDJL interacts with Hsc70 and clathrin protein

In germ cells, acrosome formation requires the combined synchronized processes of clathrin-coated vesicles’ trafficking and confluence, so the requirement of rDJL in the formation of acrosome may be due to its interaction with a specific Hsp70 and participation in the dynamics of clathrin-coated vesicles. To determine whether or not rDJL is involved in the formation of clathrin-coated vesicles, the interactions between rDJL and Hsc70 and that of rDJL and clathrin were investigated.

HEK293 cells were co-transfected with pcDNA6/V5-HisB-HA-rDJL and pEFP-C3-clathrin and harvested at 24 h later. Cell lysates were prepared and incubated with anti-HA
monoclonal antibody and the precipitated immunocomplexes separated by SDS–PAGE and the protein bands transferred to a nitrocellulose membrane. HA immunoprecipitates were immunoprobed with anti-Hsc70 antibody. As shown in Fig. 8, endogenous Hsc70 of HEK293 cell was detected in the precipitated complexes, showing an association of rDJL and Hsc70. Blots were stripped and reprobed with anti-GFP antibody. The result confirmed the occurrence of an interaction between rDJL and clathrin.

To clarify the function of rDJL in clathrin-coated vesicles, we utilized EGF receptor mediated endocytosis as model to confirm the relationship between rDJL and clathrin [19]. CHO cells were transfected with pEGFP-C3-clathrin and pDsRed1-N1-rDJL and treated with EGF. By confocal microscopy, RFP-rDJL and GFP-clathrin were found as aggregates

Fig. 7. Subcellular localization of rDJL in testicular germ cells of varying stages of differentiation. During the process of germ cell differentiation, the subcellular localization of rDJL progressed from a diffuse scattered distribution in the cytoplasm of spermatocytes to the perinuclear region of spermatids and finally to the acrosome of mature spermatozoa. Bar = 8 μm.

Fig. 8. Western blot of HEK293 proteins showing interaction between rDJL/Hsc70 protein and rDJL/clathrin protein. HEK293 cells were co-transfected with pcDNA6/V5-HisB-HA-rDJL and pEGFP-C3-clathrin (lane 1) or pcDNA6/V5-HisB-HA and pEGFP-C3-clathrin (lane 3). Cell lysates were immunoprecipitated with anti-HA antibody and the immunoprecipitated proteins immunoprobed with anti-Hsc70, anti-GFP or anti-HA antibodies. Lane 2, input of cell lysates for immunoprecipitation.

Fig. 9. CHO cells transfected with pEGFP-C3-clathrin and pDsRed1-N1-rDJL and stimulated with epidermal growth factor. Note co-localization of rDJL and clathrin proteins as spotty endocytic vesicles.
in the newly formed endosome vesicles and were co-localized with each other (Fig. 9), suggesting that rDJL formed a complex with clathrin in the formation of EGFR-mediated endocytic vesicles.

4. Discussion

Mammalian spermatogenesis is a complex phenomenon of cell differentiation involving mitotic stem cell proliferation and meiosis, followed by remodeling of haploid spermatids, progressing to the formation of mature spermatozoa. Based on the morphological features, the germ cells in various stages in the cycle located in the seminiferous epithelium can be delineated by transillumination, allowing isolation by microdissection [20–22]. In the present study, applying the method of DDRT-PCR combined with the segmentation technique of seminiferous tubules, a novel rat DnaJ like protein gene designated rDJL was isolated. The coded rDJL protein contains the highly conserved J domain, but lacks the glycine/phenylala-nine-rich sequence and the cysteine-rich zinc finger domain, indicating that it belongs to the type III subgroup of DnaJ proteins. Type I and type II DnaJ proteins have a more highly conserved J domain within their own groups and tend to interact with a broader range of substrates; whereas type III DnaJ proteins, have a lower degree of conservation and a more restricted substrate specificity [23].

rDJL is mainly expressed in testis and liver, suggesting that rDJL expression is tissue specific. Furthermore, in testis, rDJL was found only in germ cells but not in Sertoli or Leydig cells, suggesting that its expression is also cell type specific.

The earliest developmental expression of rDJL gene occurred in rat testis on the 30th postnatal day and its expression reached adult level on the 60th postnatal day and maintained at the high level subsequently. In the 60-day-old rat testis, its content in round and elongating spermatids is substantial [24,25]. Hence, the development-dependent expression pattern of rDJL indicates participation in spermiogenesis, whereby haploid spermatids undergo a series of changes in structure and function and eventually develop into mature spermatozoa. The process includes chromosome condensation, formation of acrosome and flagellum and expulsion of redundant cytoplasm.

The present immunofluorescent findings showing that rDJL is located asymmetrically in the perinuclear area of round and elongating spermatids and in the acrosome of mature spermatozoa suggest that it plays a role in the formation of the acrosome. Acrosome plays a key role during the process of sperm cell maturation, capacitation and fertilization. Thus studies on acrosome formation may uncover important clues about the mechanism of fertilizing potential of sperm. Acrosome originate from the trans-Golgi apparatus and contain hydrolytic enzymes that aid sperm in penetrating the egg’s outer membrane. The formation of acrosome is a process involving vesicle trafficking and confluence during which clathrin-coated vesicles act as the principal transport media [26]. So we hypothesized that rDJL may be a structural component of clathrin-coated vesicle. The present experimental results demonstrate that rDJL can co-immunoprecipitate with Hsc70 and clathrin protein. Furthermore, in EGF-treated CHO cells transfected with pEGFP-C3-clathrin and pDsRed1-N1-rDJL, rDJL was concentrated in the newly formed endosome vesicles and co-localized with clathrin protein. These findings suggest that rDJL is a component of the clathrin-coated vesicle and may participate in vesicular trafficking within germ cells, thus contributing to the biogenesis of acrosomes. rDJL is a small molecule and does not possess a clathrin-binding domain, indicating that the interaction of rDJL and clathrin may be indirect and probably mediated by another unidentified component.

During the process of fertilization, when a sperm contacts an egg, the content of the vesicle is released by exocytosis as the acrosomal reaction. Previous studies suggest that proteins participating in the intracellular trafficking events leading to the formation of the acrosome during mammalian spermiogenesis are also involved in regulating the acrosome reaction during fertilization [27]. The presence of rDJL in the acrosome of mature spermatozoa points to a possible function during fertilization.

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