FEBS Letters 579 (2005) 5734–5740 FEBS 30040

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

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Received 7 August 2005; revised 2 September 2005; accepted 11 September 2005

Available online 4 October 2005

Edited by Felix Wieland

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.**

Keywords: rDJL; DnaJ; Hsc70; Clathrin; Acrosome; Spermatogenesis

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 testes 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 diopters.

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Abbreviations: DDRT-PCR, differential display polymerase chain reaction; EST, expressed sequence tag; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay

0014-5793/$30.00
doi:10.1016/j.febslet.2005.09.046

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 $dT_{12}N$ (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 ddH_2O and the DNA extracted by boiling.

The isolated cDNAs were reamplified and the products denatured and dotted onto two identical nylon membranes with equal amount 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 Na_2HPO_4, 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 fragmentation of seminiferous tubule (GenBank Accession No. AF059656) was used as probe in screening a rat testis λgt10 5′-stretch cDNA library (Clontech, CA). A total of 1×10^6 clones were screened with the plaque hybridization method. Positive clones were selected and the inserts amplified, using the primers of the flank 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-^{32}P] deoxycytidine triphosphate. Autoradiography was performed to visualize the component 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 AAG CCT TTG TGC CGT-3′; The sequences of the primers for rat β-actin were: forward; 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′, and reverse; 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′.

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

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 frozen rat testis sections of 5 μm-thick were prepared and processed according to the manufacturer's instruction (Zymed Histospain-SP, MT Rabbit ACE kit, Zymed Lab. Inc. South San Francisco). After blocking with 5% BSA in 3% H_2O_2/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 H_2O_2), 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/V5-HisB-HA vector (HA tag sequence inserted into pcDNA6/V5-HisB vector between NheI and HindIII sites). HEK293 cells were transiently transfected with pcDNA6/V5-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-HSC70 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 pDsRed1-N1 vector. CHO cells were transiently transfected with pDsRed1-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