Molecular Cloning and Expression of Suppressor of Potassium Transport Defect 3 (SKD3) in Rat Testis

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Abstract. To analyze the function of heat shock protein (HSP) in spermatogenesis, rat suppressor of potassium transport defect 3 (SKD3) cDNA, a novel member of the HSP family, was cloned in rat testis, and its expression and localization was investigated in rat testis at the mRNA and protein levels. The cDNA and predicted amino acid sequence of rat SKD3 showed approximately 95.6% and 97.0% homology, respectively, with mouse SKD3. All motifs recognized in mouse were conserved in rat. SKD3 mRNA (2.3 kbs) was restricted to the testis among the tissues investigated. SKD3 mRNA was detected by in situ hybridization in spermatogonia, spermatocytes, Leydig cells, and Sertoli cells. Immunoreactive SKD3 from whole rat testis and a Leydig cell line (LC540) was seen at 76 kDa by Western blotting. SKD3 immunoreactive cells were Leydig and Sertoli cells. Although the cellular localizations of SKD3 mRNA and protein were somewhat inconsistent, rat SKD3 cDNA was characterized and various expression patterns of SKD3 mRNA and protein in rat testis were revealed in the present study.

Key words: SKD3, Rat testis, Leydig cell

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by targeting caused severe impairment of spermatogenesis [5]. Synaptonemal complexes contain HSP70.2, suggesting that HSP70.2 is involved in desynapsis during meiosis [6, 7]. New members of the HSP family and/or related proteins have also been identified [8–10], however, their precise expression patterns and functions remain to be determined. The goal of this study was to determine the expression patterns of a novel HSP (SKD3) in rat testis at both the mRNA and protein levels for further analysis of the functions of HSPs.

**Materials and Methods**

**Animals and tissue preparations**

Ten-week-old Wistar rats (240–260 g) were used throughout this study (Japan SLC, Shizuoka, Japan). To prepare the starting material for the 5′-rapid amplification of cDNA ends (5′-RACE), sampled testes were immediately processed for total RNA extraction using ISOGEN (Wako, Osaka, Japan). For Northern blot analysis, testes, ovaries, adrenal glands, kidneys, spleen, thymus, and brain were collected. All sampling was performed under pentobarbital anesthesia. Tissues were immediately frozen in liquid nitrogen until use. For in situ hybridization, rats were perfused with physiological saline, and then with Bouin’s solution through the left ventricle. Testes were excised, dehydrated in a graded series of ethanol, immersed in xylene, and embedded in paraffin wax. Sections of 5 µm thickness were prepared and processed for the procedures of in situ hybridization.

**Molecular cloning of the rat SKD3 cDNA**

To determine the sequence of full-length rat SKD3 cDNA, 5′-RACE was carried out using a 5′-RACE Core Kit purchased from Takara (Kyoto, Japan). All procedures were carried out according to the technical manual from the supplier.

**Northern blotting**

Total RNA from each tissue was extracted using ISOGEN, and poly(A)+ RNA was purified from 1 mg of total RNA sample (OligotexTM-dT, Takara, Kyoto, Japan). All procedures were carried out according to the technical manual from the supplier.

**In situ hybridization**

The method for sense and anti-sense cRNA probe preparation has been described previously [12]. An isolated rat SKD3 cDNA fragment (1954–2094) in the plasmid Bluescript was used for preparing the cRNA probe. Deparaffinized sections were treated with 4% paraformaldehyde/0.1 M phosphate buffer, 0.1 M glycine/0.2 M Tris-HCl (pH 7.6), and then with 8 mg/ml proteinase K/50 mM Tris-HCl (pH 7.5)/2 mM CaCl2, for 10 min each. Subsequently, sections were hybridized for 16 h at 45 C in the following solutions: 50% formamide, 5× standard saline citrate (SSC), 1× Denhardt’s solution, 100 mg/ml heparin, 10 mM dithiothreitol, 100 mg/ml yeast tRNA, 10% dextran sulfate, and 5 µg/ml cRNA probe. Sections were then rinsed three times in 2× SSC for 30 min at 50 C and three
times in 1× SSC at 50 C for 30 min. Sections were then incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody for 1 h at room temperature (RT) and then visualized using an enzyme-histochemical method.

**Preparation of antibody against recombinant SKD3**

Full-length SKD3 cDNA was amplified using the following primers: 5'-CAggTgTCATgATgTgTCC-3' and 5'-ggTggATgCTAgATggTg-3' (according to the rat SKD3 sequence determined in this study). Fragments were cloned into pBAD/Thio prokaryotic expression vector (Invitrogen). SKD3 was purified using Thiobond resin and enterokinase was used to immunize rabbits. The IgG fraction was obtained using a protein A column (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Western blotting**

The specificity of the antibody was confirmed by Western blotting using whole rat testis samples and a Leydig cell line (LC540, kindly supplied by HSRRB, Osaka, Japan. Its JCRB number is JCRB9064). Extracts were prepared from each homogenized tissue in a buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.125% bromophenol blue, and then boiled for 5 min. Proteins were fractionated by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS. Separated proteins were transferred onto nitrocellulose filters and blocked with 10% skimmed milk (Snow Brand Milk Products, Tokyo, Japan) and 0.5% Tween 20/PBS overnight at 4 C. Subsequently, filters were incubated with rabbit anti-SKD3 polyclonal antibody containing 0.1% SDS. Separated proteins were transferred onto nitrocellulose filters and blocked with 10% skimmed milk (Snow Brand Milk Products, Tokyo, Japan) and 0.5% Tween 20/PBS overnight at 4 C. Subsequently, filters were incubated with rabbit anti-SKD3 polyclonal antibody containing 0.5% Tween 20 for 1 h at RT, and conjugated horseradish peroxidase (HRP) goat anti-rabbit IgG for 1 h at RT. The colored reaction product was developed in 3, 3'-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-HCl (pH 7.4) containing 0.1% H2O2.

**Immunohistochemistry**

Prior to the incubation with primary antibody, tissue sections were deparaffinized in xylene, and rehydrated in a graded series of ethanol. Subsequently, sections were treated with 3% H2O2 in methanol for 10 min at RT, washed in 0.01 M PBS, then blocked with normal goat serum for 30 min at RT. Specimens were incubated with anti-SKD3 antibody overnight at 4 C. Sections were then processed with biotinylated goat anti-rabbit IgG for 30 min at RT, and washed in PBS. HRP conjugated avidin-biotin complex (Vector Laboratories, Belmont, CA, USA) was combined with secondary antibody at RT for 30 min. Immunoreactivity was visualized with 0.02% DAB in 50 mM Tris-HCl (pH 7.4) containing 0.1% H2O2.

Two controls were set up in which (1) the primary antibody was replaced by PBS or normal rabbit serum, and (2) the primary antibody was pre-incubated overnight at 4 C with an excess of the respective antigen.

**Results**

The full-length of rat SKD3 cDNA was cloned by 5’-RACE (Fig.1) and the predicted amino acid sequence was deduced (Fig. 1). The cDNA and amino acid sequences of rat SKD3 showed approximately 95.6% and 97.0% homology, respectively, with those of mouse SKD3. In the amino acid sequence, A and B motifs, which have been identified in proteins that bind ATP [13], were conserved in rat SKD3. There were four ankyrin-like consensus regions at amino acids 134–165, 166–198, 235–267, and 268–300. Notably, the amino acid sequences of the A and B motifs and two of the ankyrin-like regions (regions 1 and 4 in Fig. 1) completely matched with those of mouse.

Northern blotting revealed that expression of rat SKD3 mRNA was restricted to the testis and we could not detect signals in other organs examined, including the ovary (Fig. 2). The strongest mRNA signal detected in testis was 2.3 kbs in length. In situ hybridization also showed that SKD3 mRNA was expressed in rat testis. Signals were located in the seminiferous tubules and the interstitial tissue (Fig. 3a). In the seminiferous tubules, strong signals were detected in the first layer, i.e. types A, In and B spermatogonia, and in primary spermatocytes up to the zygotene phase. Moderately intense signals were seen in pachytenic and diplotene phases of primary spermatocytes and secondary spermatocytes. There was no signal in haploids (spermatids and spermatozoa). Regarding somatic cells, Sertoli and Leydig cells showed moderate signals (Fig. 3a). No signal was detected in controls using a sense probe (Fig. 3b). These results are summarized in Table 1.
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STOP

gcctgacccacgagggcccacagagggcagttttggagaggtgctc

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STOP

gcctgacccacgagggcccagagggcagttttggagaggtgctc
Western blotting showed immunoreactivity at approximately 76 kDa in homogenates of whole rat testis and LC540 (Fig. 4).

Immunohistochemistry showed positive reactions in Leydig and Sertoli cells (Fig. 5). No reactivity was found in spermatogenic cells. The immunolocalization of SKD3 varied in Leydig cells: the reactivity was detected widely and evenly in the cytoplasm and/or it was localized especially around the nuclear membrane. In Sertoli cells, reactivity was present in the whole cytoplasm.

Discussion

The sequence of SKD3 cDNA of rat contains not only an ATP binding domain, i.e., the A and B motifs, but also ankyrin consensus regions, and it is highly conserved between rat and mouse. Ankyrin repeats have been identified in several transcription factors [14–16] and receptors [17, 18]. Therefore, SKD3 might act similarly to these molecules and may possess ATPase activity.

In the present study, the expression of rat SKD3 mRNA was restricted to the testis. In mouse, SKD3 mRNA was expressed in various organs (and was most abundant in the testis) as shown by Northern blotting. The reason for this difference among species remains unclear, however, the examination of the expression in the ovary is the first such examination in vertebrates, and the large difference in the strength of signals between the sexes (Fig. 3) is quite interesting. In this study, we used mRNA from whole rat ovary for Northern blotting. Granulosa, thecal and interstitial cells occupy most of the volume in the ovary, and the relative volume of germ cells is quite small. Therefore, a large proportion of the mRNA from a total ovary preparation is probably derived from somatic cells. These cells of rat ovary should not express SKD3 mRNA. It is also speculated that the expression of SKD3 mRNA in rat ovary might be limited to germ cells, and its expression might therefore be undetectable by Northern blotting. The study of HSP70.2 demonstrated the sex specificity of its gene expression [6]. Also, SKD3 mRNA might be expressed in a testis-specific manner.

In situ hybridization revealed that rat SKD3 mRNA was expressed in both somatic and spermatogenic cells. Although strong signals were detected in spermatogenic cells by in situ hybridization, unexpectedly, immunohistochemistry showed no reaction in spermatogenic cells. It remains unclear whether SKD3 protein was not translated in spermatogenic cells or the present antibody was unable to recognize SKD3 translated in these cells. A detailed investigation will be required to answer this question. Consistently, positive signals were seen with in situ hybridization and immunohistochemistry in Leydig and Sertoli cells. Interesting reactivity was found around the...
nucleus of Leydig cells. SKD3 might be involved in mediating protein folding, translocation and assembly of other proteins related to the nuclear membrane of Leydig cells as a molecular chaperone.

In a study of potassium channels in spermatocytes, in situ hybridization for the mRNA of slo3, a pH-sensitive potassium channel, showed that its expression was largely restricted to spermatocytes [19]. Other channels were also localized in sperm [20–23]. It is possible that SKD3 has effects on the opening of several potassium channels.

**Table 1.** Expression patterns of SKD3 mRNA in rat testis

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Signal intensity</th>
</tr>
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<tbody>
<tr>
<td>Spermatogenic cell</td>
<td></td>
</tr>
<tr>
<td>Spermatogonium (Types A, In and B)</td>
<td>++</td>
</tr>
<tr>
<td>Primary spermatocyte</td>
<td>++</td>
</tr>
<tr>
<td>Preleptotene</td>
<td>++</td>
</tr>
<tr>
<td>Leptotene</td>
<td>++</td>
</tr>
<tr>
<td>Zygotene</td>
<td>++</td>
</tr>
<tr>
<td>Pachytene</td>
<td>+</td>
</tr>
<tr>
<td>Diplotene</td>
<td>+</td>
</tr>
<tr>
<td>Secondary spermatocyte</td>
<td>+</td>
</tr>
<tr>
<td>Spermatid</td>
<td>−</td>
</tr>
<tr>
<td>Spermatozoos</td>
<td>−</td>
</tr>
<tr>
<td>Somatic cell</td>
<td></td>
</tr>
<tr>
<td>Sertoli cell</td>
<td>+</td>
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<tr>
<td>Leydig cell</td>
<td>+</td>
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</tbody>
</table>

++: Strong, +: Moderate, –: Undetectable.

**Fig. 3.** Cellular localization of SKD3 mRNA in adult rat testis. (a) Signals of SKD3 mRNA were detected in spermatogenic cells (small arrows), Leydig cells (large arrows), and Sertoli cells (arrowheads). Bar=50 µm (b) No signal was detected using sense probes. Bar= 50 µm

**Fig. 4.** Western blotting using whole rat testis and the LC540 cell line. Immunoreactivities at 76 kDa were seen in both testis and LC540 cells.

**Fig. 5.** Immunoreactivities were found in Leydig and Sertoli cells (large arrows). In Leydig cells, reactivity was detected widely and uniformly in their cytoplasm (small arrows) and/or was localized especially around the nuclear membrane (large arrows). Bar=50 µm
channels or the transcription of their mRNAs in Leydig and/or Sertoli cells. Sertoli cells have been shown to have critical roles during spermatogenesis by studies of the spermatogenic defects of genetically modified animals [24]. The precise function of SKD3 remains to be determined, however, SKD3 may provide clues about the Sertoli cell function in spermatogenesis.

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References

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