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PROTEIN PHOSPHATASES POSSIBLY INVOLVED IN RAT SPERMATOGENESIS

Yoshinori Kitagawa[1], Kazunori Sasaki[1], Hiroshi Shima[1], Masabumi Shibuya[2], Takashi Sugimura[1] and Minako Nagao[1]*

[1]Carcinogenesis Division, National Cancer Center Research Institute
1-1, Tsukiji-5, Chuo-ku, Tokyo 104, Japan

[2]Department of Genetics, Institute of Medical Science,
University of Tokyo, 4-6-1 Shirokanedai, Minato-ku,
Tokyo 108, Japan

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Summary: The expression of mRNAs for catalytic subunits of serine/threonine protein phosphatases 1 (PP-1) and 2A (PP-2A) in various rat tissues were examined. Four kinds of probes were used to detect mRNAs for two isotypes of PP-1 (*dis*2m1 and *dis*2m2), and two isotypes of PP-2A (*PP-2Aα* and *PP-2Aβ*). mRNAs for all of these four catalytic subunits were expressed in almost all tissues at substantial levels. They were expressed in two different sizes in most tissues. Remarkable evidence is that the smaller sized mRNAs of *dis*2m1 and *PP-2Aβ*, 1.8 kb and 1.4 kb in length, respectively, were specifically highly expressed in testis. Both these smaller sized mRNAs began to be expressed at the age when meiosis started and were detected in testicular germ cells at the pachytene stage of meiotic prophase. Protein phosphatases which have peptides encoded by *dis*2m1 and *PP-2Aβ* as catalytic subunits may play important roles in spermatogenesis.

There are four major serine/threonine protein phosphatase isoenzymes in mammalian cells, PP-1, PP-2A, PP-2B (calcineurin) and PP-2C (1). They differ in metal ion requirements and sensitivities to two heat-stable protein inhibitors, inhibitor-1 and inhibitor-2 (2). The substrate specificities of PP-1 and PP-2A seem to be controlled by regulatory subunits (3-6). cDNA cloning revealed the existence of isotypes in each of the catalytic subunits of PP-1, PP-2A and calcineurin A: PP-1α and PP-1β (7), PP-2Aα and PP-2Aβ (8) and calcineurin Aα and Aβ (9). Presence of isotypes PP-2C1 and PP-2C2 in PP-2C was also found by protein purification (10).

Furthermore, two clones, *dis*2m1 and *dis*2m2 were isolated from a mouse fetal brain cDNA library as homologues of yeast PP-1. The proteins encoded by these mouse cDNAs showed about 90% identity with rabbit *PP-*

*To whom correspondence is to be addressed.

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1α (11), and *dis2*m1 and *dis2*m2 were registered as genes encoding catalytic subunits of PP-1 (11).

In this study, we examined the tissue specific mRNA expression of *dis2*m1, *dis2*m2, *PP-2Aα* and *PP-2Aβ* in rats and found unique features of *dis2*m1 and *PP-2Aβ* expression in testicular germ cells.

Materials and Methods

Northern blot analysis Total and poly(A)$^+$ RNA prepared as described (12) were fractionated in 1.0% formaldehyde-agarose gel and transferred to a nitrocellulose filter. For detection of transcripts of the two isotypes of the catalytic subunits of PP-1, 1.5 kb and 1.0 kb *Eco*RI fragments of cDNAs of *dis2*m1 and *dis2*m2 (11), respectively, were used as probes. For detection of the transcripts for the two isotypes of the catalytic subunits of PP-2A, a 0.49 kb *Pst*I-*Eco*RI fragment of rat *PP-2Aα* cDNA and a synthetic oligomer corresponding to 3' non-coding region of rat *PP-2Aβ* were used as probes (13). Hybridization was carried out at 42°C overnight in 50% formamide, 0.65 M NaCl, 0.1 M sodium PIPES (pH 6.8), 5x Denhardt's solution, 0.1% SDS, 5 mM EDTA, 10% dextran sulfate (except for the oligo-probe), salmon sperm DNA (100 μg/ml), and ^{32}P-labeled probe. All the probes were labeled with [α-^{32}P]dCTP by the random priming method (14) except the *PP-2Aβ*, oligo-probe, which was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Germ-cell fractionation Male germ cells were fractionated in a "STAPUT" unit-gravity velocity sedimentation system in a gradient of 2 to 4% (wt/vol) bovine serum albumin (15). The purity of fractions was determined by microscopic examination and fractions of more than 80% purity were pooled and used for RNA preparation.

Results

Expressions of the isotypes in various rat tissues and interstitial cell tumors of the testis

Expressions of the catalytic subunits of PP-1 in various tissues of a 6-week-old male rat were examined (Fig. 1). The mouse *dis2*m1 probe detected two mRNA species of 2.6 kb and 1.8 kb in all tissues. The 2.6 kb transcript was highly expressed in the spleen, kidney and small intestine, whereas the 1.8 kb transcript was only slightly expressed in all somatic tissues. In testis, a remarkably high level of 1.8 kb mRNA was expressed with a substantial level of 2.6 kb mRNA. A 1.5 kb transcript was detected only in low amounts in the spleen.

The mouse *dis2*m2 probe detected two mRNA species of 3.2 kb and 2.4 kb. The 3.2 kb mRNA was expressed in all tissues as a major species, and the 2.4 kb mRNA was expressed in the bone marrow and spleen as a minor species. Expression levels of *dis2*m2 were not remarkably different between tissues examined.

The *PP-2Aα* probe detected the 2.0 and 2.7 kb mRNAs as major and minor transcripts, respectively, in all tissues examined (Fig. 1), the levels of both transcripts being highest in the brain, as reported previously (16).

The *PP-2Aβ* probe also detected two species of mRNAs, which were 2.0 and 1.4 kb in size. Of the two, 2.0 kb mRNA was the major transcript in most tissues. The *PP-2Aβ* mRNA was highly expressed in the kidney, brain and testis, but very low in the bone marrow. In the testis, unlike in other tissues, the level of the 1.4 kb mRNA was higher than that of the 2.0 kb mRNA. Remarkably high levels of expression of smaller sized mRNAs of the *dis2*m1 and *PP-2Aβ* seem to be characteristics of the testis.

More than 80% of rats of the F344 strain, develop interstitial cell tumors of the testis by 2 years of age (17). Fig. 2 shows the expressions of the four catalytic subunits in normal testes and spontaneous interstitial cell tumors of the testis in which scarcely any germ cells were present. There were no remarkable differences between normal testes and interstitial cell tumors in the expressions of *dis2*m2 and *PP-2Aα*. In contrast, expression of *dis2*m1 and *PP-2Aβ* were remarkably different between normal testes and interstitial cell tumors. The 1.8 kb mRNA of *dis2*m1 and 1.4 kb mRNA of *PP-2Aβ* were abundantly expressed in all three normal testes, but only slightly in all three interstitial cell tumors. However, no remarkable or consistent changes were observed with the expression levels of 2.6 and 2.0 kb larger size mRNAs of *dis2*m1 and *PP-2Aβ*, respectively.

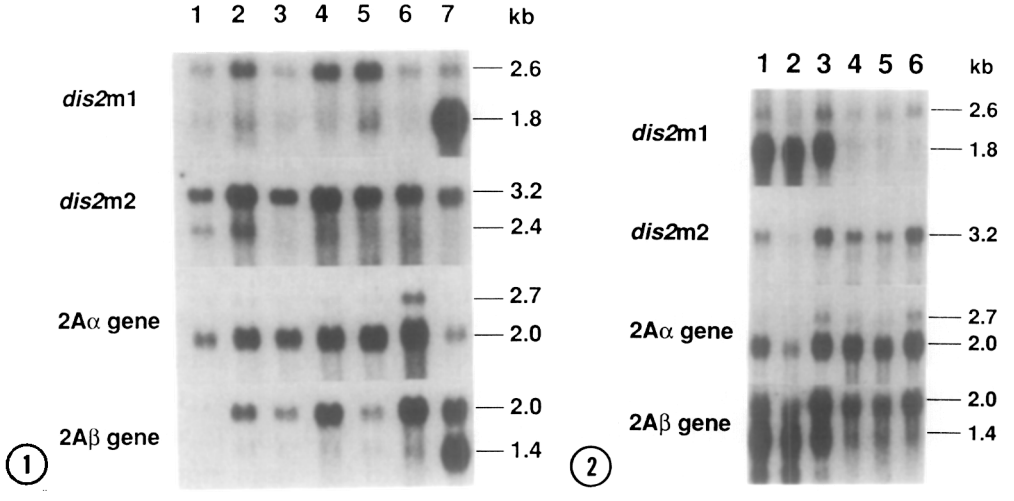


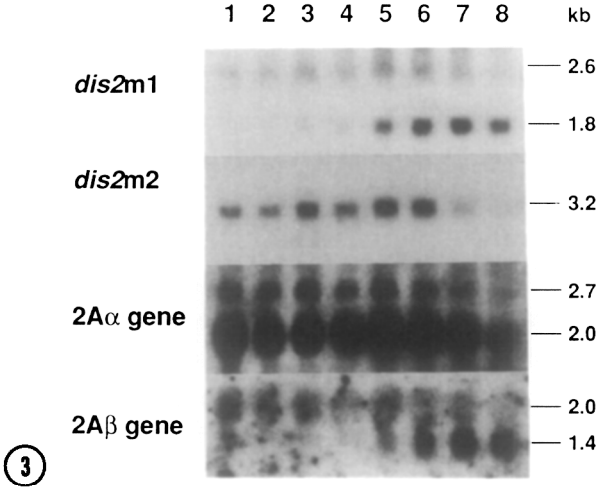
Figure 1. Tissue specific expression of catalytic subunits of PP-1 (*dis2*m1 and *dis2*m2) and PP-2A (*PP-2Aα* and *PP-2Aβ*) in a 6-week-old F344 male rat. Samples of 5 μg of poly(A)$^+$ RNA from various tissues of the rat were gel electrophoresed and transferred to a nitrocellulose filter. Probes and hybridization conditions for all experiments were as described in Materials and Methods. Lane: 1, bone marrow; 2, spleen; 3, liver; 4, kidney; 5, small intestine; 6, brain; 7, testis.

Figure 2. Expression of the catalytic subunits of PP-1 and PP-2A in rat testis and interstitial cell tumors of the testis. Samples of 10 μg of total RNA were blotted. Lanes 1 to 3, normal testis; lanes 4 to 6, interstitial cell tumors of testis. The samples in lanes 3 and 4 were obtained from the same rat.

Expressions of the isotypes during meiosis

The time courses of expression of four catalytic subunits in the testis after birth were examined to determine the involvement of these enzymes in the meiotic process (Fig. 3). In prepubertal rats, *dis2*m2 and *PP-2Aα* were mainly expressed in the testes from day 16 to 26 and their expressions decreased 5 and 9 weeks, respectively, after birth. The 1.8 kb mRNA of *dis2*m1 and the 1.4 kb mRNA of *PP-2Aβ* were expressed in the testis 24 days after birth when meiotic division occurs (18): before day 24 only trace amounts of these mRNAs were detected. The larger mRNAs of both genes were expressed continuously at low levels in rats of all ages.

To elucidate the relationship between spermatogenesis and expression of these phosphatase genes, we separated the testicular germ cells into three fractions with "STAPUT" as described in the Materials and Methods. Fraction 1 contained spermatogonia, primary spermatocytes (at the leptotene, zygotene and early pachytene stages) and secondary spermatocytes; fraction 2 consisted of primary spermatocytes at the late pachytene stage; fraction 3 consisted of round spermatids. The *dis2*m2 and *PP-2Aα* genes were expressed mainly in the late pachytene cells, whereas



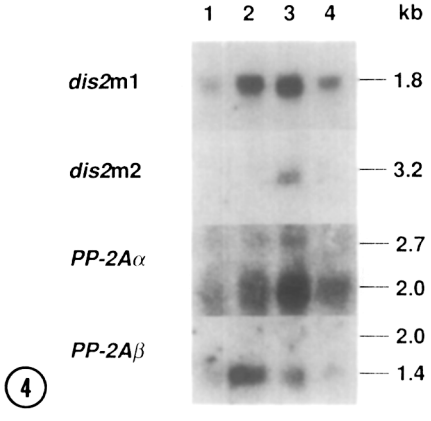


Figure 3. Time courses of expression of the catalytic subunits of PP-1 and PP-2A in rat testes after birth. Samples of 20 μg of total RNA prepared from whole testes of rats of the indicated ages were blotted. 1, 16-days; 2, 18-days; 3, 20-days; 4, 22-days; 5, 24-days; 6, 26-days; 7, 5-weeks and 8, 9-weeks.

Figure 4. Expression of the catalytic subunits of PP-1 and PP-2A in fractionated male germ cells. Samples of 20 μg of total RNA were blotted. RNAs were prepared from whole testis (lanes 1), from fractionated testicular germ cells including spermatogonia, primary spermatocytes at the leptotene, zygotene, and early pachytene stages, and secondary spermatocytes (lane 2), primary spermatocytes at the late pachytene stage (lane 3), and from round spermatids (lane 4).

*dis*2m1 and *PP-2Aβ* were expressed not only in the late pachytene stage but also in earlier stages including spermatogonia, primary spermatocytes (leptotene, zygotene, and early pachytene) and secondary spermatocytes (Fig. 4). The round spermatids also expressed these mRNAs, but at reduced levels. The 1.8 kb mRNA of *dis*2m1 and the 1.4 kb mRNA of *PP-2Aβ* which were major species in testis were proved to be major species in germ cells.

Discussion

In this study we found that *dis*2m1 and *PP-2Aβ* express mRNA at high levels with unique features in rat testis. High expression of smaller sized mRNAs of these genes chronologically coincided with the occurrence of meiotic divisions, and smaller sized mRNAs were found to exist in germ cells, at higher levels in spermatocytes than in spermatids. These results suggest that PP-1 and PP-2A having the *dis*2m1 and *PP-2Aβ* products as catalytic subunits, respectively, play important roles in spermatogenesis. Many reports which indicate the involvement of protein phosphatases in cell cycle regulation are available (11,19,20), and we think that the *dis*2m1 and *PP-2Aβ* products are possibly involved in meiosis.

Recently, Matsushime *et al.* isolated a cDNA encoding a novel kinase designated as *mak* (male germ cell-associated kinase), which is specifically expressed in testicular germ cells at the stage of meiotic division (21). The *mak* kinase shows high homology with the yeast *cdc2/CDC28* and human *cdc2*Hs products, probably being a serine/threonine kinase. Since the time of gene expression of *mak* overlaps those of expressions of *dis*2m1 and *PP-2Aβ*, cross-talks of these gene products could be expected.

Several genes, such as the human α-tubulin gene (22), the A kinase regulatory subunit genes (23), and the c-*raf* gene (24) express shorter mRNAs in the testis, as compared with other tissues. The mechanisms underlying production of short mRNA are alternative splicing (25), alternative poly(A) addition (26) and use of a different promoter (22). The isotype switch during maturation of the testis was observed on the gene located on the X chromosome (27). This is also a possible mechanism involved in expression of testis specific mRNA. Elucidation of the mechanisms involved in production of shorter mRNAs of *dis*2m1 and *PP-2Aβ* are in progress.

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