Differentiation-dependent enhanced expression of protein phosphatase 2Cβ in germ cells of mouse seminiferous tubules

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Abstract The presence of five distinct isoforms of protein phosphatase 2Cβ (PP2Cβ-1 ~ -5) is known. In this study, we demonstrate that the mRNA levels of PP2Cβ-3, -4 and -5 and PP2Cβ protein level increased during the course of the first wave of spermatogenesis in neonatal mouse testis. Northern blot and in situ hybridization analyses revealed that PP2Cβ-3, -4 and -5 were expressed predominantly in pachytene spermatocytes and in more highly differentiated germ cells. The substrate specificity of PP2Cβ-4 determined with artificial substrates differed from those of PP2Cβ-3 and -5, suggesting that the difference in the structure of PP2Cβ-3, -4 and -5 reflect their unique physiological functions in testicular germ cells.

Key words: Protein phosphatase 2C; Isoform; Spermatogenesis; Testicular germ cell

1. Introduction

Protein phosphorylation plays pivotal roles in the regulation of cellular functions, and protein phosphatases in combination with protein kinases control the phosphorylation levels of cellular proteins. Protein phosphatase 2C (PP2C) is one of the four major protein serine/threonine phosphatases (PP1, 2A, 2B and 2C) in eukaryotic cells and is distinct from the other three protein phosphatases because of its absolute requirement for Mg^{2+} or Mn^{2+} and because its amino acid sequence shows little similarity to the sequences of the other phosphatases [1].

Two distinct gene products of PP2C (α and β) are reported to be present in various mammalian tissues [2–4]. In addition, the isolation of cDNA clones encoding five distinct isoforms of mouse PP2Cβ (PP2Cβ-1, -2, -3, -4 and -5) has been also reported [4–7]. It is likely that these isoforms are the products of alternative splicing of a single pre-mRNA, since they have a common 5′ terminal region and differ only in their 3′ terminal regions which encode 11–24 amino acids. They also differ in the tissue specificity of their expression [5–7]. Genes encoding PP2C homologs have also been isolated from other eukaryotic cells [8–14].

Recent investigations of the functions of mammalian PP2C have revealed that PP2C participates in the regulation of AMP-activated protein kinase activity [15], Ca^{2+}-dependent signal transduction [16] and DNA repair systems [17]. However, the specificity of the physiological functions of each PP2C isoform in mammalian cells has yet to be elucidated.

In a previous study using reverse transcriptase polymerase chain reaction (RT-PCR), we demonstrated that PP2Cβ-3, -4 and -5 are expressed specifically in adult mouse testis and intestine and that the expression levels of these three PP2Cβ isoforms in newborn mouse testis are much lower than in adult mouse testis [7]. In this study we investigated the relationship between the expression of PP2Cβ isoforms and testicular maturation. We found that the expression levels of PP2Cβ-3, -4 and -5 increase during the course of the first wave of spermatogenes in mouse testis and that they are specifically expressed in pachytene spermatocytes and more highly differentiated germ cells of the seminiferous tubules. We also present evidence which suggests that PP2Cβ-4 have substrate specificities distinct from those of PP2Cβ-3 and -5.

2. Materials and methods

2.1. Isolation of RNA

Artificial cryptorchid testis of mice were produced as described previously [18]. Fractionation of mouse testis was performed according to a previously described method [19,20]. Germ cell fractions were prepared from ICR mice and Sertoli cell and Leydig cell fractions were from jsd/jsd mice. Preparation of total RNA from mouse testis was performed using the acid guanidium thiocyanate phenol chloroform method. Poly(A)$^+$ RNA was isolated from 500 μg of total RNA using oligotex-dT(30) < Super > (Takara Shuzo, Kyoto, Japan).

2.2. Northern blot analysis

The probes used were *Eco*RI–*Sma*I fragment of pTK-1 (1.2 kbp, common to PP2Cβ-1, -2, -3, -4 and -5) labeled using the random primed labeling method with [α-^{32}P]dCTP, or anti-sense oligonucleotides specific to PP2Cβ-3, -4 and -5 (ttggtaggtcggtcggcctctgctttgtcaccagagttctcagct) labeled by a polynucleotide kinase reaction using [γ-^{32}P]ATP.

2.3. In situ hybridization

A cDNA fragment containing the 3′ terminal region of pTK-4 (*Eco*RI–*Eco*RI 0.6 kb fragment of RT-PCR product-2) was subcloned into Bluescript SK. This construct was linealized with *Bam*HI for the antisense probe or *Hin*dIII for the sense probe and transcribed in vitro using T7 (for the antisense probe) or T3 (for the sense probe) RNA-polymerase to generate digoxygenin-11-UTP-labeled single strand RNA probes. In situ hybridization was performed as described previously [21].

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2.4. Preparation of antibody

A polyclonal rabbit antibody against mouse PP2Cα was prepared using purified recombinant mouse PP2Cα as the antigen. A polyclonal rabbit antibody against a synthetic mouse PP2Cβ oligopeptide (TTNEDFRAADKSGSALE) conjugated to hemocyanine was prepared and affinity purified using a standard method. The anti-PP2Cα and -PP2Cβ antibodies were named AB 103 and AB 106, respectively.

2.5. Immunoblot analysis

Whole testes from 10-day-old or adult mice were homogenized in a 20 mM Tris-HCl buffer (pH 6.9) containing 2% (v/v) glycerol, 5 mM 2-mercaptoethanol and proteinase inhibitors (2 μg/ml pepstatin, 2 μg/ml antipain, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 0.1 mM benzamidine, 1 mM EGTA, 1 mM PMSF, 0.1 mM TPCK and 0.5 mM TLCK) and centrifuged at 3600×*g* for 15 min at 4°C and the resultant supernatants were submitted to immunoblot analysis. AB 103 or AB 106 rabbit antibody (diluted 1:200) was used as the first antibody. Color development was achieved with 0.1 M Tris-HCl (pH 9.0) containing BCIP and NBT.

2.6. Construction of plasmids for expression of GST-PP2Cβ fusion proteins

The plasmid vector pGEX-2T was used for the expression of GST-PP2Cβ-3, -4 and -5 fusion proteins. The *Nco*I–*Sca*I fragment was cut out from the expression plasmid pKMC-TK-3, -4 and -5, which harbored PP2Cβ-3, -4 and -5 [7]. The *Nco*I site of each of these fragments was given a blunt end by a Klenow fragment and ligated into pGEX-2T which had previously been digested by *Sma*I; the resultant plasmid was named pGEX-PP2Cβ-3, -4 and -5.

2.7. Expression of GST-PP2Cβ fusion proteins in E. coli cells and preparation of PP2Cβ proteins

Escherichia coli cells (JM109) were transformed by the expression plasmids (pGEX-PP2Cβ-3, -4 and -5), and the transformants were cultured in Luria-Bertani medium containing ampicillin (50 μg/ml) at 30°C. When the OD_{600} reached 0.1, IPTG was added to a final concentration of 1 mM and the cells were cultured for 12 h. Then the cells were harvested, resuspended in a solution containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl, 50 μg/ml lysozyme and the proteinase inhibitors already described. After incubation for 15 min on ice, cells were sonicated for tw 15-s bursts (28 kHz) using a Model UR-20 sonicator (Tomy Seiko, Tokyo, Japan) and centrifuged at 3600×*g* for 15 min and the resultant supernatants were saved. The GST-fusion proteins in the cell extracts were adsorbed to glutathione agarose beads and treated with thrombin to generate recombinant PP2C proteins. Protein determination was performed by Bradford's method using bovine serum albumin as the standard [22].

2.8. Assay of protein phosphatase activities

Phosphorylation of α-casein by casein kinase II and whole histone by protein kinase A using [γ-^{32}P]ATP was performed essentially as described previously [23]. Casein kinase II was purified from bovine thymus using a previously described procedure [24]. Protien kinase A was purified as described previously [23]. Protein phosphatase activity was measured in the presence or absence of 10 mM $MgCl_2$ and 1 μM okadaic acid. Protein phosphatase activity was assayed by the release of [^{32}P]phosphate from [^{32}P]phosphohistone or [^{32}P]phosphocasein essentially as described previously [23].

3. Results and discussion

3.1. Expression of PP2Cβ mRNA during the course of testicular maturation

It has been established that the first wave of spermatogenesis takes place in a synchronized manner in mouse testis during the first 20-day period after birth. Therefore, we first determined, by Northern blot analysis, whether the expression

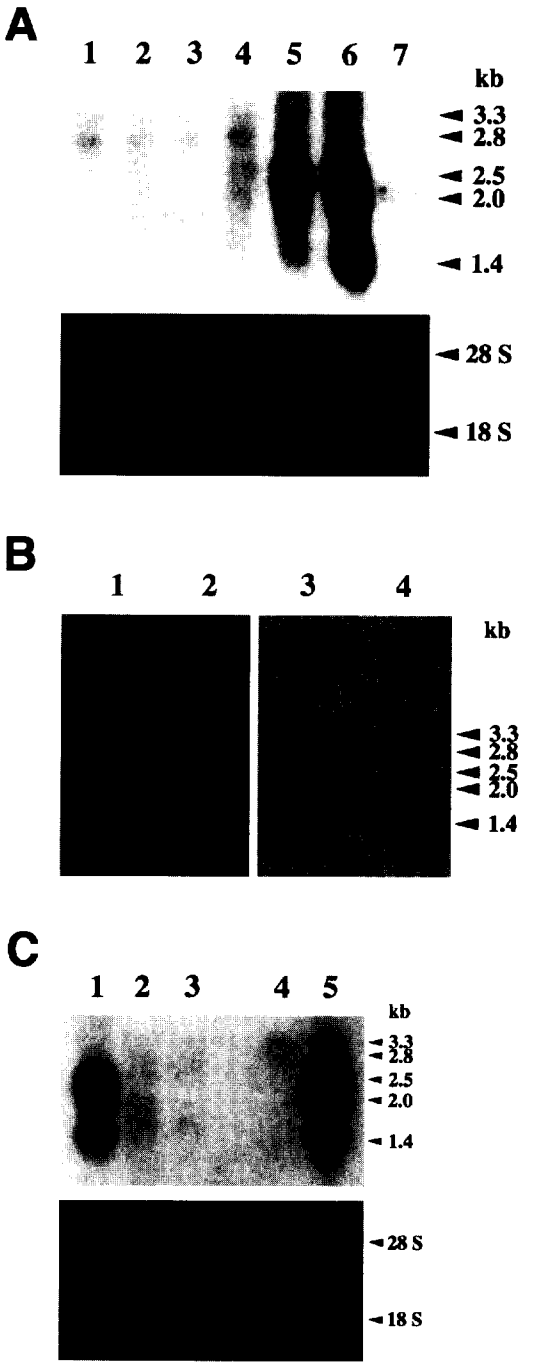

Fig. 1. Expression of PP2Cβ mRNAs in the course of testicular maturation. A: Aliquots (25 μg) of total RNA fractions extracted from the testes of 2-, 6-, 10-, 16- or 24-day-old mice (lanes 1–5, respectively), and from 8-week-old (lane 6) or cryptorchidism (lane 7) mice were examined by gel electrophoresis. Northern hybridization was performed using a ^{32}P-labeled *Eco*RI–*Sma*I fragment (1.2 kbp) of the pTK-1 insert as the probe. B: Aliquots (5 μg) of a poly(A)$^+$ RNA fraction (lanes 1 and 3) and a 10 μg aliquot of a rRNA fraction (oligotex-dT nonadsorbing fraction, lanes 2 and 4) extracted from testes of 8-week-old mice were examined by gel electrophoresis. Northern hybridization was performed using a ^{32}P-labeled antisense oligonucleotide specific to PP2Cβ-3, -4 and -5 (lanes 1 and 2) or an *Eco*RI–*Sma*I fragment (1.2 kbp) of the pTK-1 insert (lanes 3 and 4) as the probe. C: Aliquots (15 μg) of total RNA fractions from germ (lane 1), Sertoli (lane 2) and Leydig (lane 3) cell fractions of testes from 10-week-old mice and of whole testis from 7-day-old (lane 4) and 10-week-old (lane 5) mice were examined by gel electrophoresis. Northern hybridization was performed using a ^{32}P-labeled *Eco*RI–*Sma*I fragment (1.2 kbp) of the pTK-1 insert as the probe.

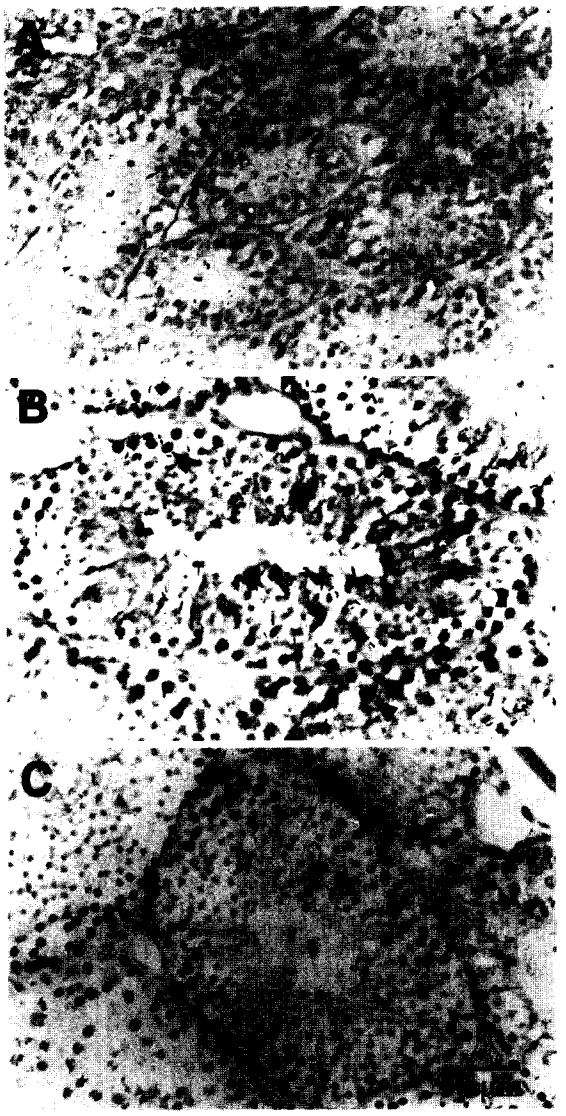

Fig. 2. In situ hybridization of neonatal and adult mice testes. In situ hybridization of testes from 7-day-old (A) and adult (B and C) mice was performed using antisense (A and B) and sense (C) ribonucleotide probes common to PP2Cβ-3, -4 and -5. The arrowhead indicates a pachytene spermatocyte. The line indicates 50 μm length.

levels of PP2Cβ isoforms alter during the course of this first wave of spermatogenesis. The probe used in this experiment was a cDNA fragment of a region common to the five PP2Cβ isoforms. As shown in Fig. 1A, only two faint mRNA bands of 3.3 kb (PP2Cβ-1) and 2.8 kb (undetermined mRNA related to PP2Cβ), were observed in neonatal mouse testis until 10 days after birth (Fig. 1A, lanes 1–3). In addition to these mRNA signals, a diffuse signal of 2.0∼2.5-kb mRNA appeared on day 16 and thereafter (Fig. 1A, lanes 4–6). An additional 1.4-kb mRNA signal was also observed on day 24 and in adulthood (Fig. 1A, lanes 5 and 6). In contrast, no bands were detected in artificial mouse cryptorchid testis, which had type A spermatogonia but no germ cells in more advanced stages of differentiation (Fig. 1A, lane 7).

The enhanced levels of 1.4- and 2.0∼2.5-kb mRNAs in adult testis are consistent with our previous study in which we demonstrated using RT-PCR that the mRNA levels of PP2Cβ-3, -4 and -5 in adult mouse testis were much higher than those in newborn mouse testis.

3.2. Determination of sizes of PP2Cβ-3, -4 and -5 mRNAs

In order to identify the mRNA signals corresponding to PP2Cβ-3, -4 and -5 mRNAs, Northern blot analysis was performed with poly(A)$^+$ RNA from adult testis using an oligonucleotide specific for a common region of the mRNAs encoding PP2Cβ-3, -4 and -5, which is not present in the PP2Cβ-1 and -2 mRNAs, as the probe. As shown in Fig. 1B, this probe hybridized to the 2.0∼2.5-kb mRNA, but not to the 1.4-kb mRNA (lane 1). Therefore we concluded that the 2.0∼2.5-kb mRNA signal observed in adult testis corresponded to PP2Cβ-3, -4 and/or -5, but that the 1.4-kb mRNA signal was unrelated to these three isoforms of PP2Cβ. These results also suggest that the 1.4-kb mRNA encodes a novel isoform of PP2Cβ the expression of which is also enhanced during the course of testicular maturation.

In order to characterize the 1.4-kb mRNA signal, Northern blot analysis was performed with oligotex-dT adsorbing and nonadsorbing fractions of total RNA from adult testis using a cDNA fragment of a region common to the five isoforms of PP2Cβ as the probe (Fig. 1B, lanes 3 and 4). Interestingly, a substantial level of 1.4-kb mRNA signal was detected in the oligotex-dT nonadsorbing fraction, but the signal was faint in the oligotex-dT adsorbing fraction (poly(A)$^+$ RNA fraction). Therefore, this mRNA may not have a poly(A)$^+$ tail or may have a tail that is not long enough to be adsorbed to the oligotex-dT.

3.3. Expression of PP2Cβ-3, -4 and -5 in testicular cell fractions

We then studied the cell specificity of the expression of PP2Cβ-3, -4 and -5 in adult testis. Northern blot analysis was performed with the total RNAs from three separate testicular cell fractions of adult mice (germ cells, Sertoli cells and Leydig cells) (Fig. 1C). The 2.0∼2.5- and 1.4-kb mRNA signals were detected in the germ cell fraction but not in Sertoli and Leydig cell fractions (Fig. 1C, lanes 1–3). These results and the observation that the 2.0∼2.5- and 1.4-kb mRNA signals were not observed in the total RNA fraction from cryptorchid testis (Fig. 1A, lane 7) demonstrated that these isoforms were specifically expressed in germ cells.

In order to identify the cells which express PP2Cβ-3, -4 and -5, we performed in situ hybridization of testes of 7-day-old and adult mice using PP2Cβ-3, -4 and -5 specific antisense riboprobe. No positive signal was observed in the testes of 7-day-old mice (Fig. 2A). However, a substantial amount of positive signal was observed in pachytene spermatocytes, and a faint signal was also observed in more highly differentiated germ cells, in testes of adult mice (Fig. 2B). On the other hand, no positive signal was detected when a sense riboprobe was employed (Fig. 2C).

3.4. Expression of PP2C proteins in testes of newborn and adult mice

We then performed Western blot analysis to determine whether the total amount of PP2Cβ protein increases in accordance with the enhanced mRNA levels of the PP2Cβ isoforms during the course of testis maturation. The antibodies used in this experiment, AB 103 and AB 106, were specific for PP2Cα and PP2Cβ, respectively, and no cross-reactivity was

observed when immunoblot analysis was performed with purified recombinant PP2Cα and PP2Cβ proteins (data not shown). No difference in the total amount of PP2Cα protein was observed between testes of 10-day-old and adult mice (Fig. 3, lanes 1 and 2). In contrast with PP2Cα, PP2Cβ protein in 10-day-old testis was hardly detectable on the Western blot analysis, but a substantial amount of PP2Cβ protein was detected in adult testis (Fig. 3, lanes 3 and 4). These results strongly suggest that the expression levels of the PP2Cβ-3, -4 and -5 proteins increase as the mRNA levels of these PP2Cβ isoforms increase in the course of the testis maturation.

Next, we tested whether total PP2C activity in the extracts of testis increased in parallel with the enhanced PP2Cβ protein level. No difference in the Mg^{2+}-dependent and okadaic acid-insensitive protein phosphatase activity was observed between crude extracts of testes from 10-day-old and adult mice (data not shown). We do not know why the PP2C activity of the extract from testes of adult mice was not higher than that from newborn mice. This may be due either to the presence of a much larger amount of PP2Cα than PP2Cβ in mouse testis or to the presence of a strict regulatory system of PP2C activity in the cells. These possibilities have not been tested because a system for the differential assay of PP2Cα and PP2Cβ has not been established.

3.5. *Substrate specificity of PP2Cβ isoforms*

In order to test the possibility that the three PP2Cβ isoforms differ in their substrate specificities and thereby play distinct physiological roles in vivo, we determined their substrate specificities using artificial substrates. As shown in Fig. 4, the relative activity ratios (activity for ^{32}P-labeled histone/activity for ^{32}P-labeled casein) of PP2Cβ-3 and -5 were about 4–6-times higher than that of PP2Cβ-4. In fact the molecular activities of PP2Cβ-3, -4 and -5 for ^{32}P-labeled casein were similar but the activities of PP2Cβ-3 and -5 for ^{32}P-labeled casein were about 4–6-times as high as that of PP2Cβ-4 (data not shown). Since the carboxyl terminal regions of PP2Cβ-3 and -5 share a unique common sequence of 15 amino acids containing four successive hydrophobic amino acids at the carboxyl terminal end (FYQPSIAYSDNVFLL [7]) which is not present in PP2Cβ-4, variation in the carboxyl terminal regions of PP2Cβ isoforms may be related to their substrate specificities in vivo.

The expression of unique isoforms of PP1, 2A and 2B in mammalian testis has also been reported [25–27]. In the present study the specific expression of PP2Cβ-3, -4 and -5 in germ cells of the seminiferous tubules has been demonstrated

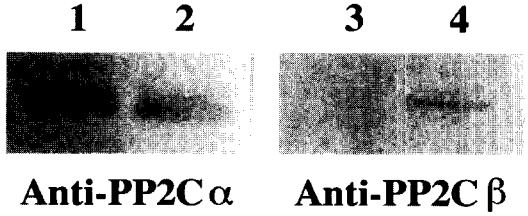

Fig. 3. Expression of PP2Cα and PP2Cβ in neonatal and adult mice testes. Aliquots (15 μg) of protein extracted from the testes of 10-day-old (lanes 1 and 3) and 8-week-old (lanes 2 and 4) mice were resolved by SDS-PAGE on a 10% (w/v) gel and blotted onto a nitrocellulose membrane. Immunostaining was performed using anti-PP2Cα (lanes 1 and 2) and anti-PP2Cβ (lanes 3 and 4) polyclonal antibodies.

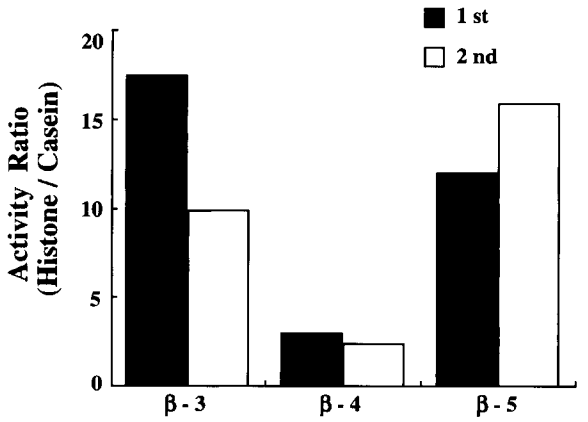

Fig. 4. Substrate specificity of recombinant PP2Cβ isoforms. The relative activity ratios (activity for ^{32}P-labeled histone/activity for ^{32}P-labeled casein) of the purified recombinant PP2Cβ-3, -4 and -5 are depicted. The results represent two separate experiments.

for the first time. Taken together, these lines of evidence strongly suggest that the functions of the seminiferous tubules are regulated by unique phosphorylation/dephosphorylation reactions. Therefore, to clarify the physiological significance of the presence of testis-specific molecular species of protein phosphatase, the physiological substrates of each of these phosphatases must be identified.

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