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Expression and Localization of Prolyl Oligopeptidase in Mouse Testis and Its Possible Involvement in Sperm Motility

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ABSTRACT—Prolyl oligopeptidase (POP) expression in mouse testis during sexual maturation was examined. Northern blot analysis showed that POP mRNA expression was highest at 2 weeks of age, and gradually reduced thereafter. However, enzyme activity was almost constant during the examined period. *In situ* hybridization study revealed a change in the expression site of POP mRNA in testis during sexual maturation. Positive signals were detected in all types of cells in the seminiferous tubules before maturation, and were restricted to spermatids at the spermatogenesis cycle stages I–VIII in adult mice. POP was detected in the insoluble fraction of sperm by Western blot analysis. Immunohistochemical analyses showed that POP is localized in the spermatids at steps 12–16 of spermiogenesis and in the midpiece of the sperm flagellum. It was also found that specific POP inhibitors, poststatin and benzyloxycarbonyl-proline-proline, suppressed sperm motility. These results suggest that POP may be involved in meiosis of spermatocytes, differentiation of spermatids, and sperm motility in the mouse.

Key words: mouse, testis, prolyl oligopeptidase, gene expression, sperm motility

INTRODUCTION

Prolyl oligopeptidase (POP; EC3.4.21.26; prolyl endopeptidase, a post-proline cleaving enzyme) is a widely distributed serine endopeptidase catalyzing the hydrolysis of the peptide bond, -Pro-X-. POP has been purified and cloned from many species (Ishino *et al.*, 1998; Rennex *et al.*, 1991; Shirasawa *et al.*, 1994; Amin *et al.*, 1999; Ohtsuki *et al.*, 1994; Sattar *et al.*, 1990; Yoshimoto *et al.*, 1987; Yoshimoto *et al.*, 1991). POP may play roles in many biological processes, such as the maturation and degradation of peptide hormones and neuropeptides (Mentlein, 1988; Wilk, 1983), learning and memory (Cunningham and O'Connor, 1997a), DNA synthesis (Ishino *et al.*, 1998; Ohtsuki *et al.*, 1997), cell differentiation (Ohtsuki *et al.*, 1994), and signal transduction (Williams *et al.*, 1999). However, conclusive results have not yet been reported, and the physiological role of POP remains to be elucidated. Furthermore, in spite of its wide distribution, the function of POP has been examined only in limited tissues and organs.

The male reproductive tract plays many important roles in reproduction, to which spermatogenesis and androgen production are essential. A number of proteinases are thought to participate in these processes. POP could be one

such proteinase based on its relatively high expression in testis and sperm. In fact, Yokosawa *et al.* (1983) purified POP from ascidian sperms for purpose of biochemical characterization. More recently, Yoshida *et al.* (1999) isolated POP from herring testis, and their findings suggested that POP exists on the surface of the sperm tail and interacts with the herring sperm-activating protein. These results led to the idea that POP could also have some function in the mammalian male reproductive system. In this study, we examined POP expression and localization in mouse testis during postnatal development. We also report the occurrence of POP in sperm and the suppression of sperm motility by specific POP inhibitors.

MATERIALS AND METHODS

Animals

Mice (male, strain C57BL/6Crj) were obtained from Charles River Inc., Yokohama, Japan. The animals were kept under controlled conditions (25°C; 14-hr light and 10-hr dark) and allowed free access to food and water. The animals were sacrificed by cervical dislocation, and the tissues were rapidly removed, frozen in liquid N₂, and stored at -80°C until use. For some experiments, the tissues were used immediately.

RNA isolation

Total RNAs were isolated from the testes of the mice at 2 to 8 weeks of age using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions.

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Northern blot analysis

Twenty micrograms of the total RNAs were electrophoresed on a formaldehyde/agarose gel and transferred to a Nytran membrane (Schleicher and Schuell, Dassel, Germany). The blot was hybridized for 16 hr with a [³²P]-labeled 262-bp *Hind*III-*Bam*HI fragment of mouse POP cDNA, which is included in exon 15 (Kimura *et al.*, 1999), at 42°C in 50% formamide, 5×Denhardt's solution, 5×0.15 M NaCl/8.65 mM NaH₂PO₄/1.25 mM EDTA (SSPE), 1% SDS, and 100 µg/ml salmon sperm DNA. The membrane was washed at 50°C in 0.1% SDS/0.1×SSC and exposed to Kodak Biomax film. Mouse 18S ribosomal RNA (18S rRNA) oligonucleotide probe, 5'-CGGCATGTATTAGCTCTAGAATTACCACAG-3', was used as an internal standard (Raynal *et al.*, 1984).

Expression and purification of recombinant POP

The expression vector for mouse POP was constructed by inserting the complete open reading frame of its cDNA into the *Eco*RI site of pET30 (Novagen, Madison, WI). The inserted region was amplified by PCR with a primer set of 5'-CCGGAATTCATGCTGTCCTTCCAGTACCC-3' and 5'-CCGGAATTCCTTACTGGATC-CACCTCGATGT-3' using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The sequence of the product was confirmed by DNA sequencing. The construct was transformed to *E. coli* strain BL21(DE3)pLys (Novagen), and the cells were grown at 37°C in Luria-Bertani medium until the optical density at 600 nm reached 0.5. Isopropyl β-D(-)-thiogalactopyranoside was added to reach a final concentration of 1 mM, followed by incubation at 37°C for an additional 5 hr. The cells were harvested by centrifugation and disrupted by sonication and freeze-thawing in 50 mM Tris-HCl (pH 7.3) (THB). The collected supernatant was applied to a DEAE-cellulose (DE-52) column (50 ml bed volume) and the column was washed extensively with THB. The POP was eluted with 50 ml each of THB and the same buffer containing 0.2 M NaCl (THBN). Fractions with POP activity were pooled and applied to a Ni-column (2 ml bed volume; Invitrogen, Carlsbad, CA). After the column had been washed extensively with THBN, the POP was eluted with THBN containing 50 mM histidine. SDS-PAGE analysis of the POP preparation thus obtained showed a single band, the molecular mass of which was calculated to be 75kDa.

Preparation of POP antibody

In this study, two mouse POP antibodies were prepared using rabbits. The purified recombinant POP was used as the antigen. Specific antiserum was raised by injecting each of 2 female rabbits (2 months old) with 0.2 mg each of the antigen emulsified with Freund's complete adjuvant. Boosting started 2 weeks later, by injection at 2-week intervals with 0.2 mg of the antigen emulsified with Freund's incomplete adjuvant. The antisera were obtained from blood collected after three booster injections. Anti-mouse POP antibody was also raised against a synthetic peptide (YPQQDSGKSDGTETSTNLHQKLCYHVLGTDQ) corresponding to amino acids 190–219 in the mouse POP (Ishino *et al.*, 1998). Three female rabbits (2 months old) were immunized with the peptide coupled to keyhole limpet hemocyanin (Sigma Genosys Japan, Hokkaido, Japan). Animals were injected with 0.2 mg of the antigen emulsified with Freund's complete adjuvant. Two weeks after the first injection, rabbits were boosted at 2-week intervals with 0.2 mg of the antigen emulsified with Freund's incomplete adjuvant. The antisera were obtained from blood collected after five booster injections.

Preparation of mouse testis extract and POP activity assay

Testes from 2- to 8-week-old mice were homogenized with a Polytron homogenizer in ice-cold phosphate-buffered saline (PBS). The homogenates were centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatants were used for the enzyme assay. POP activity toward succinyl(Suc)-Gly-Pro-methylcoumaryl-7-amide(MCA) was assayed as previously described (Kimura *et al.*, 1998).

Western blot analysis

Samples of mouse testis extracts were separated by SDS-PAGE (Laemmli, 1970) and transferred to polyvinylidene difluoride membrane (Towbin *et al.*, 1979). The blotted membrane was incubated with rabbit anti-mouse POP antiserum at 1:200 dilution. The membrane was further incubated with biotinylated anti-rabbit IgG antibody and subsequently with avidin conjugated with horseradish peroxidase. Immunoreactive signals were detected using an ECL Western blot detection kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer's protocol.

In situ hybridization

Testes from 2- to 8-week-old mice were frozen in isopentane-dry ice and cut into 10-µm sections on silane-coated slides. The sections were fixed with 4% paraformaldehyde (Wako Pure Chemicals, Osaka, Japan) in PBS for 10 min, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/HCl (Sigma, St. Louis, MO). After prehybridization at room temperature for 6–24 hr in 50% formamide, 6 × SSPE, 5 × Denhardt's solution, and 500 µg/ml yeast transfer RNA, the sections were incubated for 18 hr at 55–65°C in the same buffer containing 100–200 ng/ml digoxigenin (DIG)-labeled probes. The probes were prepared by *in vitro* transcription of a 262-bp *Hind*III-*Bam*HI fragment of mouse POP cDNA with T3 or T7 RNA polymerase. The hybridized sections were washed three times in 0.2 × SSC at 55–65°C for 20 min per wash. Signals were detected with a DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany).

Immunohistochemistry

Frozen sections (10 µm) of adult C57BL/6 mouse testis were dried, fixed in methanol at –20°C for 20 min, treated with 3% H₂O₂ in PBS, and blocked with BlockAce (Dainippon Seiyaku, Tokyo, Japan) for 1 hr at room temperature. Then, sections were incubated with POP peptide antiserum (1/5000) for 18 hr at 4°C, followed by incubation with horseradish peroxidase conjugated anti-rabbit IgG (Amersham Pharmacia Biotech.) for 1 hr at room temperature. Immunocomplexes were detected using a diaminobenzidine detection kit (Vector laboratories, Burlingame, CA).

Sperm suspensions obtained from the cauda epididymis of 8-week-old mice were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and placed onto a Superfrost microslide glass with silane coating. After washing with PBS twice, the non-specific binding was blocked with BlockAce. For indirect immunofluorescent staining, the slides were incubated with POP peptide antiserum (1/1000) for 18 hr at 4°C, treated with fluorescein isothiocyanate-labeled anti-rabbit IgG (Sigma) for 1 hr at room temperature, washed with PBS, and observed under a fluorescent microscope (Olympus, Tokyo, Japan). In some experiments, sperm suspensions were processed as described above except that the permeabilization treatment with 0.1% Triton X-100 was omitted.

Sperm preparation and motility assays

The cauda and caput of the epididymis were isolated from male mice and immersed in pre-warmed PBS with various reagents. The reagents used were two specific inhibitors of POP, poststatin and benzyloxycarbonyl-proline-proline (Z-Pro-Prolinal). The tissues were minced in the solutions and incubated at 37°C for 20 min. For the sperm motility assays, the percentage of motile sperm was determined using a hemocytometer to count the samples in varied order. For preparation of sperm, the samples were incubated without the reagents and filtered with a cell strainer (100 µm, Falcon). After centrifugation at 300 × *g* for 5 min at room temperature, the sperm precipitate was suspended in PBS and the supernatant was collected by centrifugation at 10,000 × *g* for 10 min at 4°C. The resulting supernatant was the 'secreted fraction.' The sperm was disrupted by sonication and centrifuged at 12,000 × *g* for 10 min at 4°C. The precipitate was the 'insoluble fraction' and the supernatant was the 'soluble fraction.'

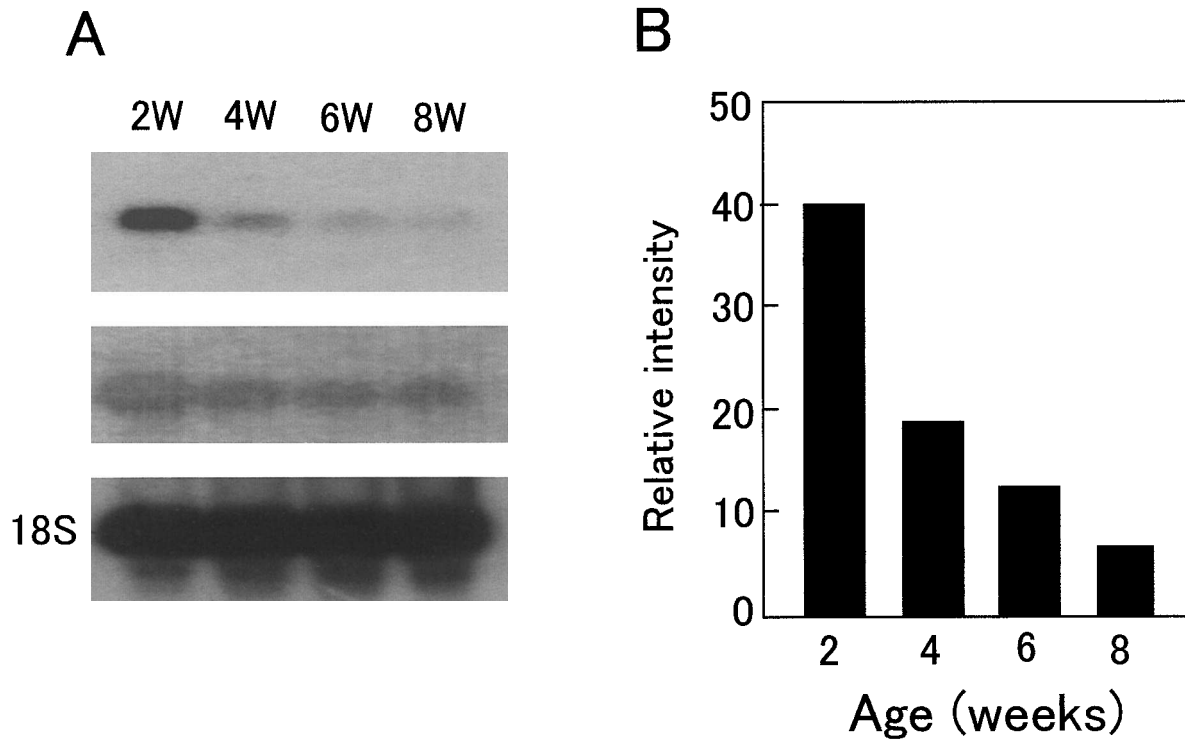


Fig. 1. POP mRNA expression in mouse testis during sexual maturation.

A, Total RNAs were isolated from testes of 2-, 4-, 6-, and 8-week-old mice and used for Northern blot analysis. The probe used was a [32 P]-labeled 262-bp fragment of mouse POP cDNA. The signals were detected by exposing the film for 1 day (upper panel) or 3 days (middle panel). The lower panel represents the level of the control 18S ribosomal RNA. **B**, Signal intensities of the bands shown in the upper panel of **A** were quantified by densitometric analysis using NIH Image (National Institute of Health, Bethesda, MD), and data from the bands were normalized with the control 18S ribosomal RNA signals.

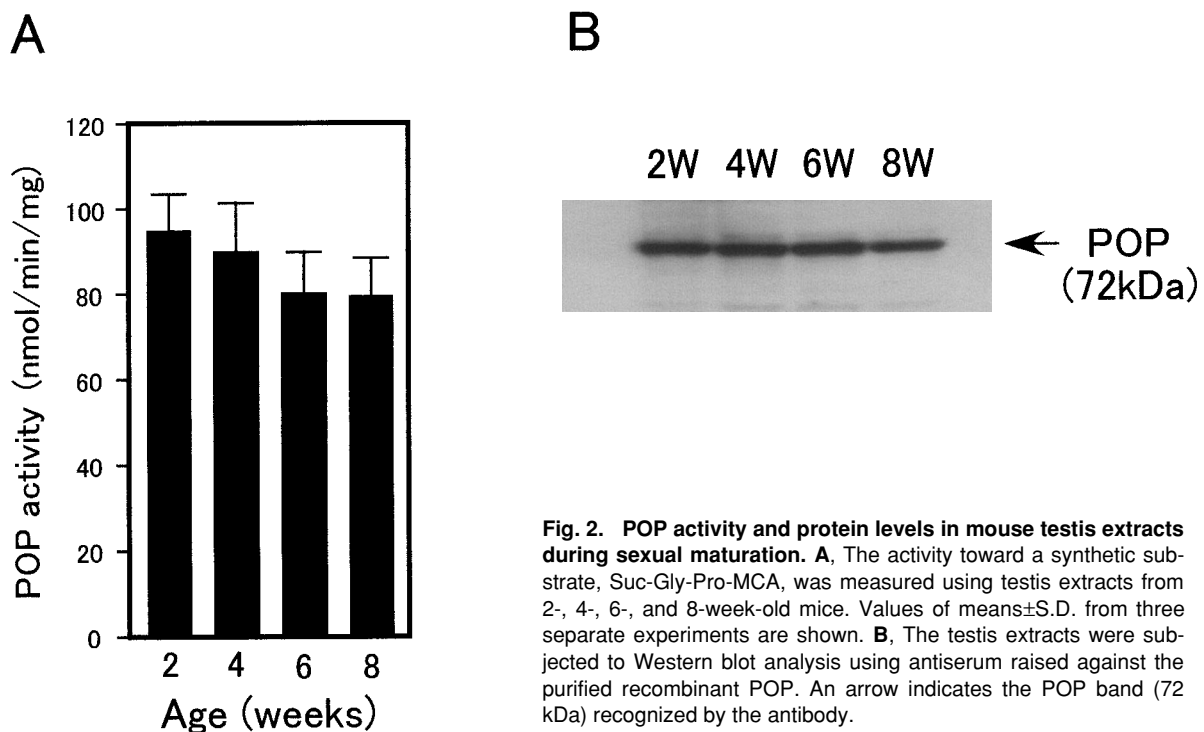


Fig. 2. POP activity and protein levels in mouse testis extracts during sexual maturation. **A**, The activity toward a synthetic substrate, Suc-Gly-Pro-MCA, was measured using testis extracts from 2-, 4-, 6-, and 8-week-old mice. Values of means \pm S.D. from three separate experiments are shown. **B**, The testis extracts were subjected to Western blot analysis using antiserum raised against the purified recombinant POP. An arrow indicates the POP band (72 kDa) recognized by the antibody.

RESULTS

Expression of POP mRNA in mouse testis during sexual maturation

Northern blot analysis was conducted using total RNAs

from testes of 2- to 8-week-old mice to investigate POP mRNA expression during the sexual maturation of mouse. A single POP band was detected at 2.8 kb in all lanes (Fig. 1A). The POP signal was the strongest in testis from 2-week-old mouse, and became weaker as the animals grew

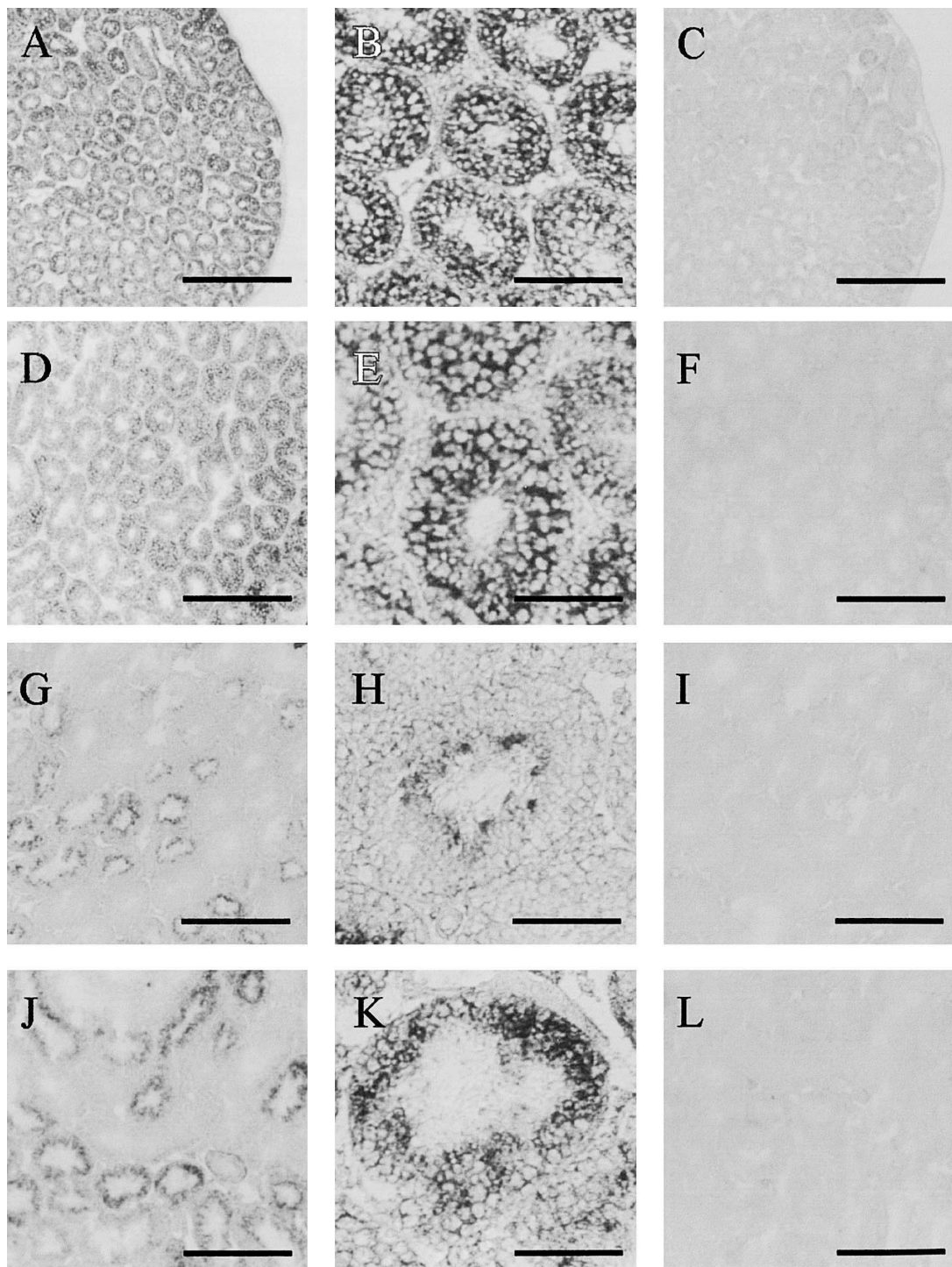


Fig. 3. *In situ* detection of POP mRNA in mouse testes. Mouse testis sections from 2- (A, B, and C), 4- (D, E, and F), 6- (G, H, and I), and 8-week-old mice (J, K, and L) were hybridized with DIG-labeled antisense (A, B, D, E, G, H, J, and K) or sense probes (C, F, I, and L). The positive signals were detected by alkaline phosphatase activity. The probe used was a 262-bp *HindIII*-*Bam*HI fragment of mouse POP cDNA. Bars represent 10 μ m in B, E, H, and K, and 50 μ m in all others.

to maturity. In the testes from 8-week-old mouse, the POP signal was clearly detected only when the membrane was exposed to the film for comparatively a long time. The signal strength was quantified and normalized by 18S ribosomal RNA, the results of which are summarized in Fig. 1B. The relative strength of POP signals at 2 weeks was 40, but those at 4, 6, and 8 weeks were drastically reduced; the POP mRNA level at 8 weeks was about 1/8 that of young 2-week-old mice. These results indicate that POP mRNA was expressed strongly in testis from 2-week-old mouse, and that expression gradually weakened as the animal matured.

POP activity in mouse testis during sexual maturation

The POP activity in testes from 2- to 8-week-old mice was measured using a specific substrate, Suc-Gly-Pro-MCA, as described in the MATERIALS AND METHODS section. As shown in Fig. 2A, the POP activity was almost constant among the testes of these mice, though it tended to decrease from 4 weeks to 6 weeks. Western blot analysis was performed to confirm the POP protein expression, and only a single band was detected at 72 kDa in each lane (Fig. 2B). These results indicate that, in contrast with remarkable changes in the relative POP mRNA content, protein and activity levels in mouse testis are fairly constant during sexual maturation.

Localization of POP mRNA in mouse testis

POP mRNA localization was examined by the *in situ* hybridization method. Testes from 2- to 8-week-old mice were sectioned and hybridized with DIG-labeled sense and antisense probes for mouse POP. The results showed that the POP mRNA localization in testis changed during sexual maturation. In the testis of 2-week-old mouse, when primary spermatocytes reached an early pachytene stage and no spermatids had appeared, the cells in the seminiferous tubule were all positive, whereas the Leydig cells were negative (Fig. 3, A and B). In the testis of 4-week-old mouse, when all types of cells appeared and the spermatogenesis cycle had just started, all the cells in the seminiferous tubules were positive (Fig. 3, D and E). In the testes of 6- and 8-week-old mice, when the mice had completely matured and the spermatogenesis cycle was operating, spermatids gave specific positive signals for POP, though not in all seminiferous tubules (Fig. 3, G, H, J, and K). No significant signals were detected in other cells including Leydig cells, Sertoli cells, spermatogonia, and spermatocytes. In order to identify the stage of the spermatogenesis cycle in the POP-positive tubules, we counterstained the sections with methylgreen. In the POP-positive tubules, round spermatids were stained, indicating that POP mRNA was restricted to the spermatids at stages I–VIII of the spermatogenesis cycle (Oakberg, 1956). No positive signals were detected using the sense probe in any case (Fig. 3, C, F, I, and L). These results indicate that POP mRNA localization becomes restricted during sexual maturation.

Immunohistochemical examination with anti-POP anti-serum

Immunohistochemical staining of a frozen section of

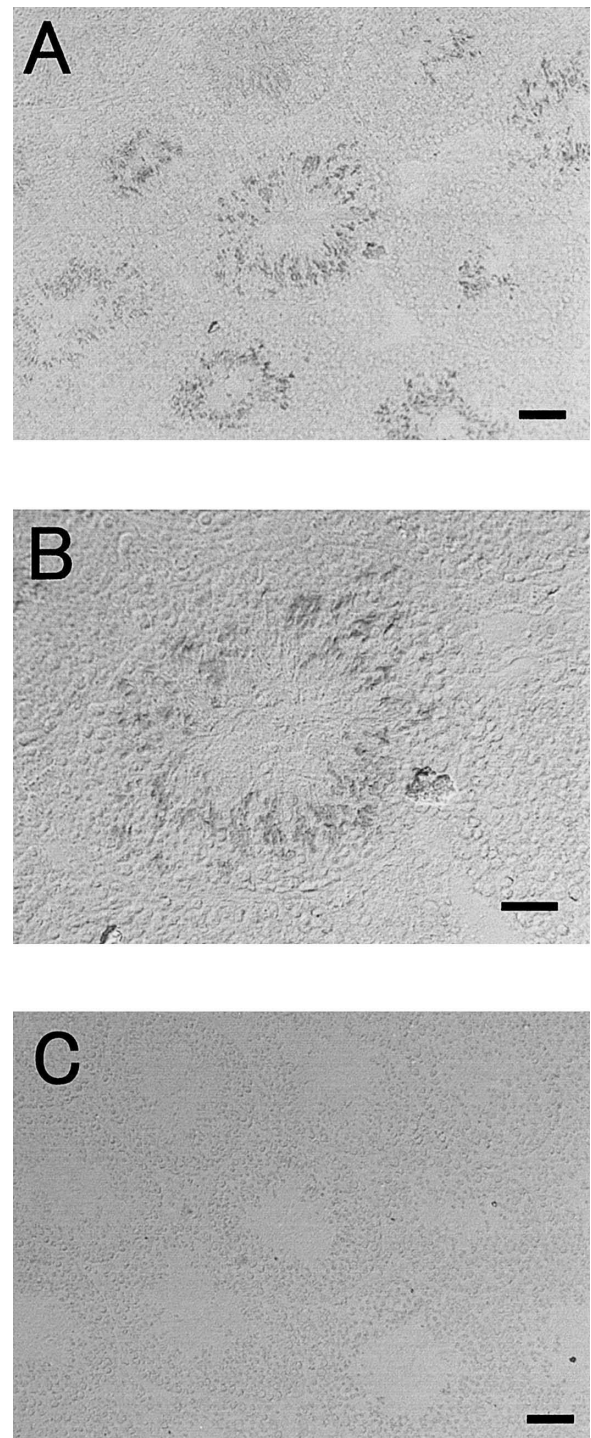


Fig. 4. Immunohistochemical staining of POP in mouse testis. Testicular frozen sections were fixed with methanol. The sections were incubated with diluted antiserum raised against the synthetic POP peptide (A and B) or preimmune serum (C), and then with horseradish peroxidase-conjugated anti-rabbit IgG. Immunocomplexes were detected by diaminobenzidine. Bars represent 20 mm in A and C, and 5 mm in B.

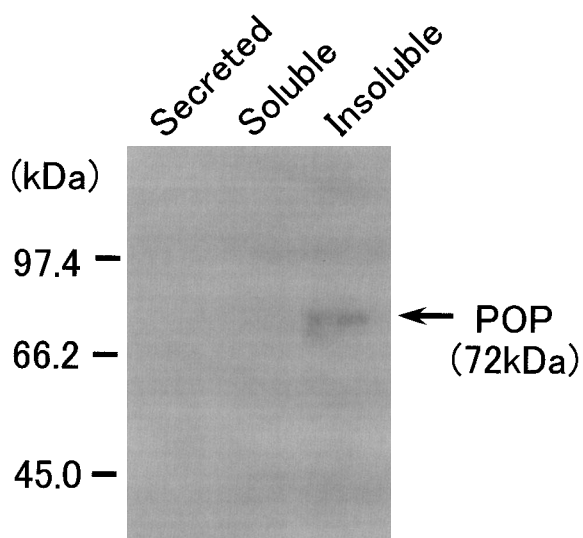


Fig. 5. Immunological detection of POP in mouse sperm. Mouse sperm was fractionated into secreted, soluble, and insoluble fractions as described in the **MATERIALS AND METHODS** section. The samples were electrophoresed using a 10% SDS-PAGE gel and the signal was detected with antiserum raised against the recombinant POP protein. The arrow at the position corresponding to 72 kDa indicates the POP signal. The positions of the molecular size markers are shown to the left.

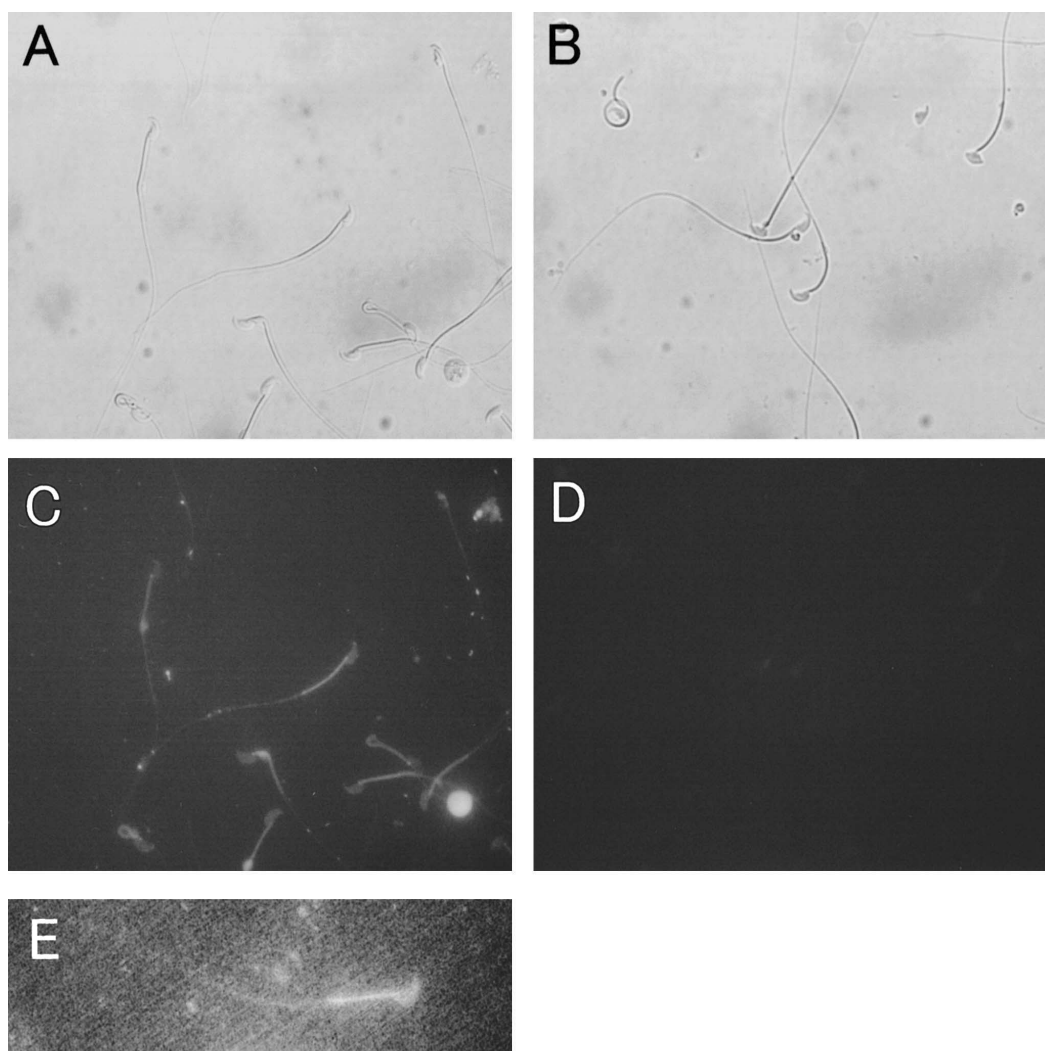


Fig. 6. Localization of POP in mouse sperm. Sperms were isolated from the cauda epididymis of 8-week-old mouse. Sperm preparations were immunostained with anti-POP peptide antiserum (**A**, **C**, and **E**) or preimmune rabbit serum (**B** and **D**). Immunofluorescent microscopic images (**C**, **D**, and **E**) and phase-contrast microscopic images (**A** and **B**) are shown. An enlarged view of a representative sperm stained with the antibody is shown in **E**.

adult mouse testis was performed using the anti-POP peptide antiserum. As shown in Fig. 4, a positive signal was present in germ cells but not in Sertoli cells or Leydig cells. From the result of counterstaining with methylgreen, POP protein localization was determined to be associated with elongating spermatids at step 12 and elongated spermatids at steps 13 to 16 of spermiogenesis cycle. However, round spermatids at steps 1 to 7 and elongating spermatids at steps 8 to 11 showed no staining. The intensity of the staining seemed constant during spermiogenesis. In the frozen testis section, the heads of elongated spermatid stained. To determine the precise location of POP protein expression, fixed sperms isolated from the cauda epididymis were stained using the same POP peptide antiserum (Fig. 5). Intense positive staining was detected in the midpiece of the sperm flagellum. In addition, significant signals were also observed in the sperm head. The tail region of the sperm flagellum gave very weak signals. Similar experiments were conducted using sperms without permeabilization treatment with 0.1% Triton X-100. We obtained essentially the same results as those with permeabilized sperms (data not shown).

POP expression in mouse sperm

POP expression in the mouse sperm was further investigated. Three fractions were prepared from sperms isolated from mouse cauda epididymis: the buffer, which had been used for the activation of sperm, containing proteins or peptides secreted from the sperms, and the soluble and insoluble fractions, respectively, that had been generated by sonication of sperms. Western blot analysis was conducted to detect POP in these fractions. As shown in Fig. 6, the POP signal was detected at 72 kDa only in the insoluble fraction. We measured for POP enzyme activity using these fractions. No significant activity was detected in either the sperm activation buffer or the soluble fraction. On the other hand, the insoluble fraction showed detectable POP activity (data not shown).

In a separate experiment, we assayed for POP activity using fresh sperm suspensions. The sperm suspension, which had been prepared after washing twice with PBS, gave POP activity of 0.59 and 5.00 nmol/min/ 1.6×10^5 sperms for Suc-Gly-Pro-MCA and Suc-Gly-Pro-Leu-Gly-Pro-MCA, respectively. PBS recovered from the second washing showed only 1/4 the activity of the sperm-associated enzyme activity for both substrates.

The results described above clearly indicate that POP is present exclusively in the insoluble fraction of the mouse sperm and that POP activity is indeed detectable in fresh sperms. The data suggest that POP localization in sperm rendered its active site susceptible to the substrates.

Effects of POP inhibitors on sperm motility

Effects of POP inhibitors on sperm motility were examined. Sperms freshly isolated from mouse cauda epididymis were incubated in the buffer containing either Z-Pro-Prolinal

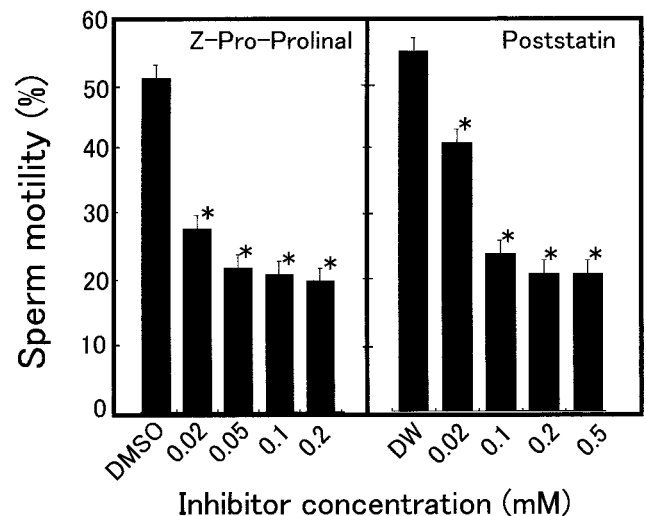


Fig. 7. Effects of POP inhibitors on mouse sperm motility. Mouse sperm was activated in PBS containing Z-Pro-Prolinal (left panel) or poststatin (right panel) at the indicated mM concentrations. The motile sperm was counted with a hemocytometer and the percentages are shown. DMSO and distilled water (DW), solvents of Z-Pro-Prolinal and poststatin, respectively, were used as controls. Values of means \pm S.D. from three separate experiments are shown. * Significantly lower than the control value ($P < 0.05$).

or poststatin. The percentage of motile sperm in each ouabain solution was counted and compared with the percentage of motile sperm in the buffer alone (Fig. 7). The results revealed that 0.02 mM Z-Pro-Prolinal and 0.1 mM poststatin reduced sperm motility to about half that of the control. These results indicate that POP might play an important role in the motility of mouse sperm.

DISCUSSION

In the present study, we showed that POP mRNA expression in mouse testis is the strongest at 2 weeks and gradually weakens as the mouse matures. This observation is consistent with the histological localization of POP mRNA determined by *in situ* hybridization study; POP mRNA is localized in all cells in the seminiferous tubules at 2 and 4 weeks, whereas it is restrictedly localized in spermatids at stages I-VIII, at 6 and 8 weeks. On the other hand, the specific activity of POP was found to be rather constant through at all periods examined. There are several possible explanations for such differences in the postnatal expression patterns of POP mRNA and protein. (1) There might be another enzyme(s) that carries POP activity, as suggested by previous reports (Cunningham and O'Connor, 1997b; Matsubara *et al.*, 1998). This enzyme would interfere with accurate estimation of POP activity, particularly in extracts from 4- to 8-week-old mouse testis. (2) Existence of the endogenous inhibitors of POP in the testis extracts of 2- to 4-week-old mice might have resulted in underestimation of enzyme activity. Indeed, endogenous inhibitors of POP have been reported from bovine brain (Ohmori *et al.*, 1994), rat brain

(Soeda *et al.*, 1985), and porcine pancreas (Yoshimoto *et al.*, 1982). (3) Differential turnover rates of POP mRNA and protein in the testis might be a cause of the difference in the postnatal developmental pattern. At this time, it is not clear which explanation is most plausible.

Postnatal development of mouse testis has been well characterized morphologically (Bellvé *et al.*, 1977; Goetz *et al.*, 1984). The diploid spermatogonia proliferate at 3–4 days postpartum (dpp); meiotic spermatocytes appear at about 10 dpp; haploid spermatids begin to differentiate at about 20 dpp; and the mature spermatozoa are first released at about 35 dpp. The stage of 2 weeks used in this study is the time at which the spermatocytes have just appeared, and at 4 weeks the spermatogenesis cycle has just started. During these periods, POP mRNA was expressed in all the cells in the seminiferous tubules. This finding tempts us to speculate that POP is involved in the meiosis of the spermatocytes and in the differentiation of the spermatids. Such speculation seems to be consistent with previous reports that POP may play a role in DNA synthesis (Ohtsuki *et al.*, 1997; Ishino *et al.*, 1998) and differentiation (Ohtsuki *et al.*, 1994), although the detailed mechanism underlying its role has not yet been elucidated.

Spermiogenesis shows the most dramatic morphological changes, including formation of the head and flagellum, condensation of the nucleus, formation of the acrosome, and elimination of excess cytoplasm as a residual body. In the present study, we showed the expression of POP mRNA in association with the round spermatids (steps 1 to 8 of spermiogenesis) at stages I–VIII of the spermatogenesis cycle. On the other hand, the localization of the POP gene product in adult mouse testis was found in the spermatids at steps 12–16 of spermiogenesis cycle. These findings strongly suggest that POP plays an important role in spermiogenesis.

An interesting finding in the current study is that POP was also detected in the sperm from mouse cauda epididymis. Detection of POP in the sperm was rather surprising because POP is generally thought to be a soluble cytoplasmic protein, and sperms have little cytoplasm. Since sonication of sperms did not generate a soluble form of POP at a detectable level, it is likely that POP is tightly associated with the membranous and/or nuclear materials of sperms. In this context, we should note that previous studies have reported the occurrence of a membrane-associated form of POP. Shirasawa *et al.* (1994) detected both soluble and membrane-bound forms of the enzyme in human T-cells. More recently, novel POP enzymes associated with synaptosomal membranes of bovine brain (O'Leary and O'Connor, 1995; O'Leary *et al.*, 1996) and microsomal membranes of young rat liver (Matsubara *et al.*, 1998) have been documented. In addition, Ishino *et al.* (1998) demonstrated the cytoplasmic and nuclear localization of POP in Swiss 3T3 cells.

Immunohistochemical analysis of the sperm, performed using specific POP antibody, revealed that localization of

the protein was predominantly in the midpiece of the sperm flagellum. The current observation that clear signals were seen even without permeabilization treatment with 0.1% Triton X-100 strongly suggests that POP is probably localized in the native sperm in such a manner that extracellularly added antibody is easily accessible to the molecule. It should be noted that the antibody used for the current histological study was raised against a synthetic peptide corresponding to the mouse POP amino acid sequence 190–219 (Ishino *et al.*, 1998). A recent study by Fülöp *et al.* (1998) established the three-dimensional structure of porcine muscle POP. The protein contains a peptidase domain with an α/β hydrolase fold, and the active site is covered by the central tunnel of a unique β -propeller (Fülöp *et al.*, 1998). Assuming that the three-dimensional structure of mouse POP is highly similar to the established structure of porcine POP, the above 30-residue peptide is considered to constitute a β -propeller domain, consisting of seven similar propeller structures of POP. From these considerations, it is reasonable to assume that at least one of the seven β -propeller domains of POP is perhaps exposed to the extracellular environment. Further, we found that suspensions of native mouse sperms exhibited POP activity. This finding is consistent with the idea that the peptidase domain of the enzyme is also situated extracellularly. However, further examinations are necessary to confirm this idea.

Taking into consideration that the midpiece of the flagellum is important for flagellar movement of the sperm, we examined whether POP was involved in sperm motility. For this purpose, POP inhibitors, such as Z-Pro-Prolinal and poststatin, were tested in a sperm motility assay, and were found to significantly reduce the motility of mouse sperms. Given that previous studies have clearly established that both inhibitors are highly specific for this enzyme (Wilk and Orłowski, 1983; Aoyagi *et al.*, 1991), we tentatively presume that POP plays a role in the flagellar movement of mouse sperm. Previously, the implication that POP is involved in sperm motility has been reported for the Pacific herring (Yoshida *et al.*, 1999). In that species, sperms are virtually immotile and only become motile after they make contact with egg-derived substances (Morisawa *et al.*, 1992; Yanagimachi *et al.*, 1992). Herring sperm-activating proteins (HSAP) have been isolated and found to be homologous to Kazal-type trypsin inhibitors (Oda *et al.*, 1995, 1998). More recently, both POP inhibitors and substrates were found to inhibit the activation of sperm motility by HSAP (Yoshida *et al.*, 1999). These results, together with other biochemical data, give rise to the possibility that POP is a candidate for the receptor of HSAP in herring sperm. In contrast to herring sperms, mouse sperms are motile in the absence of any egg-derived substances, and mouse sperm motility is inhibited with POP inhibitors. In this regard, the involvement of POP in motility seems largely different between mouse and herring sperms. The role of POP in sperm motility of the mouse is obscure at this time.

In this study, we investigated the expression and local-

ization of POP in mouse testis. To the best of our knowledge, this is the first report demonstrating that in adult mouse testis POP is localized in spermatids and spermatozoa in a membrane-associated manner. In addition, evidence for the possible involvement of POP in sperm motility of the mouse is presented herein. Although the precise physiological role of POP remains to be elucidated, our findings could provide a basis for future studies of POP in the male reproductive organ.

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