Molecular cloning and characterization of a novel dual-specificity protein phosphatase possibly involved in spermatogenesis

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Dual-specificity protein phosphatases (DSPs) play roles in the regulation of mitogenic signal transduction for extracellular stimulation and the cell cycle. In the present study, we identified a novel DSP, termed TMDP (testis- and skeletal-muscle-specific DSP). Nucleotide sequence analysis of *TMDP* cDNA indicated that the open reading frame of 597 bp encodes a protein of 198 amino acid residues with a predicted molecular mass of 22.5 kDa. The deduced amino acid sequence contains a motif for a conserved catalytic domain of DSPs and shows highest similarity to human *Vaccinia* HI-related phosphatase (45.5 % identity) but low homology to the mitogen-activated protein kinase phosphatase and CDC25 subfamilies of DSPs. Recombinant TMDP protein exhibited intrinsic phosphatase activity towards both phospho-seryl/threonyl and -tyrosyl residues of myelin basic protein, with similar specific activities *in vitro*. Northern-blot analysis revealed that *TMDP* is most abundantly expressed in the testis. The expression in the testis is characterized as follows: (i) *TMDP* mRNA first appeared 3 weeks after birth, corresponding to the time that meiosis begins; (ii) *TMDP* mRNA was abundant in fractionated spermatocytes and round spermatids; and (iii) hybridization *in situ* showed that the *TMDP* mRNA is localized in spermatocytes and/or spermatids in seminiferous tubules. These data demonstrate that TMDP is a novel DSP abundantly expressed in the testis and suggest that TMDP may be involved in the regulation of meiosis and/or differentiation of testicular germ cells during spermatogenesis.

Key words: cDNA, DSP, skeletal muscle, testis.

INTRODUCTION

Protein phosphatases are involved in the regulation of cell proliferation and differentiation by co-operation with protein kinases [1]. Protein phosphatases consist of two families with different substrate specificities towards phospho-seryl/threonyl or -tyrosyl residues [2]. Dual-specificity protein phosphatases (DSPs) constitute a new family of protein tyrosine phosphatases (PTPs) characterized by the ability to dephosphorylate phospho-seryl/threonyl residues in addition to phospho-tyrosyl residues. Since *Vaccinia* VH1 was first reported as a DSP [3], an increasing number of DSPs have been identified by cDNA cloning. Most of them are reported to dephosphorylate both phospho-threonyl and -tyrosyl residues in the T-X-Y motif of the mitogen-activated protein kinase family, and to cause inactivation of their kinase activity. They include mitogen-activated protein kinase phosphatase (MKP)-1 (CL100/3CH134/erp) [4–6], human *Vaccinia* HI homologous phosphatase-2 (hVH-2)/MKP-2/threonine-tyrosine phosphatase-1 (TYP-1) [7–9], hVH-3/B23 [10,11], hVH-5/M3-6 [12,13], MKP-3/rVH-6/PYST1 [14–16], MKP-X/PYST2 [14,16], MKP-4 [17] and phosphatase of activated cells-1 (PAC-1) [18]. The phosphatase activity of CDC25 is required essentially for entry into mitosis through dephosphorylation of CDC2 [19]. *Vaccinia* HI-related phosphatase (VHR) [20] has a well-characterized catalytic mechanism [21–23] and is suggested to be involved in the regulation of meiotic maturation in *Xenopus* oocytes [24].

Spermatogenic cells involved in spermatogenesis are located in the seminiferous tubules, in which a variety of biological events such as proliferation of spermatogonia, meiotic division of spermatocytes and maturation of spermatids to spermatozoa are processed. Although a regulatory mechanism of spermatogenesis is still unclear, protein phosphorylation seems to be involved as an important molecular mechanism in the regulation of spermatogenesis because several protein kinases and phosphatases are expressed specifically in the testis [25–34]. Recently, it was reported that target disruption of the protein phosphatase $PP1\gamma$ gene, which encodes an isoform of type-1 serine/threonine protein phosphatase, induces meiotic defect in the testis [35].

In the present study, we have identified a novel DSP, termed TMDP (testis- and skeletal-muscle-specific DSP). The protein phosphatase activity of TMDP protein and the stage-specific expression of *TMDP* mRNA during spermatogenesis are examined and discussed in relation to physiological roles.

MATERIALS AND METHODS

cDNA cloning

By screening the expressed-sequence-tag database (dbEST) for a novel dual phosphatase, a partial mouse cDNA clone (accession no. AA497918) showing similarity with human VHR [20] was identified. A partial cDNA fragment of the novel dual phosphatase was obtained by reverse transcriptase (RT)-PCR using

Abbreviations used: DSP, dual-specificity protein phosphatase; TMDP, testis- and skeletal-muscle-specific DSP; PTP, protein tyrosine phosphatase; dbEST, expressed-sequence-tag database; RT, reverse transcriptase; 5′ RACE, rapid amplification of 5′ cDNA ends; GST, glutathione S-transferase; pNPP, *p*-nitrophenyl phosphate; MBP, myelin basic protein; Tyr(P)-MBP, tyrosine-phosphorylated MBP; Ser/Thr(P)-MBP, serine/threonine-phosphorylated MBP; MKP, mitogen-activated protein kinase phosphatase; VHR, *Vaccinia* HI-related phosphatase.

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The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB027003 and AB027004.

primer 1, 5′-CGCTCTGCCACAATTGTCTTGG-3′, and primer 2, 5′-AGGATCTCTGGAAACAGCAGAG-3′, according to the sequence of the dbEST clone described above. The first-strand cDNA was synthesized by SuperscriptII (Gibco-BRL) using oligo(dT) as a primer. Total RNA from the mouse testis was used as a template. The amplified 267-bp fragment was cloned into the pCRII vector (Invitrogen) and sequenced. To obtain a full-length cDNA, rapid amplification of 5′ cDNA ends (5′ RACE) was carried out using the 5′ RACE system from Gibco-BRL. Experimental procedure for 5′ RACE was according to the manufacturer's instructions. Briefly, the first-strand cDNA was synthesized from total RNA of the 10-week-old mouse testis or skeletal muscle using primer 2. The specific antisense primers, primer 3, 5′-GTGCTCCCTAATTCAGAGTC-3′, and primer 4, 5′-CCTGGAGCTGTCGGAGGAAGC-3′, were used for nested PCR. The amplified products were cloned into pCRII and sequenced. The full lengths of the testis-type or skeletal-muscle-type *TMDP* cDNA were also cloned by RT-PCR from total RNA of the 10-week-old mouse testis or skeletal muscle using the following primers: primer 3 and primer 5, 5′-CCGAATT-CGAATGGACTCGCTACAGAAGCAGG-3′ or primer 6, 5′-GCAGACCTGACCGCAGGCAGAG-3′, respectively, which were specific for *TMDP*. The PCR product was cloned into pCRII and sequenced. Sequence of the full-length cDNA obtained by RT-PCR was identical to that of 5′ RACE products. The positions of primers 1–6 are presented in Figure 2(a) (see below).

Fractionation of spermatogenic cells

The testes of 10-week-old mice were dissociated by treatment with collagenase/dispase and filtered through nylon mesh. Enriched populations of primary spermatocytes, round spermatids and elongated spermatids were obtained by unit gravity sedimentation in a CelSep chamber containing a 2–4 % linear Pertol gradient in PBS. The purities of spermatocytes, round spermatids and elongated spermatids were about 80, 80 and 75 %, respectively.

Northern-blot analysis

Samples of 20 μg of total RNA were separated on a 1 % agarose gel containing 17 % formaldehyde, blotted on to nitrocellulose membranes (Schleicher & Schuell) and fixed by UV irradiation. The membranes were hybridized with a ^{32}P-labelled 267-bp fragment (nucleotides 430–696 in Figure 2b, see below) of *TMDP* cDNA in hybridization buffer [50 % formamide/1 × Denhardt's solution/5 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/50 mM NaH_2PO_4 (pH 6.5)/1 % glycine/100 μg/ml boiled salmon sperm DNA] at 42 °C overnight. The membranes were then washed twice with 2 × SSC/0.1 % SDS at room temperature for 5 min followed by 0.1 × SSC/0.1 % SDS at 50 °C for 15 min. The bands were visualized using a BAS 1000 imaging analyser (Fuji Photo Film) or by exposure to X-Omat X-ray film (Kodak).

Mutagenesis *in vitro*

Cys-138 at an active site of TMDP was mutated to Ser by PCR-based mutagenesis as described previously [36]. Primary PCR generated two DNA fragments containing the mutation. Full-length *TMDP* cDNA was used as a template for primary PCR with two sets of primers: set 1, primer 5 and primer 7 (5′-CTCACCCCCATAGCGGAGTGGACCAGCACTC-3′), and set 2, primer 8 (5′-TCCACTCCGCTATGGGGGTGAG-3′) and primer 3. The underlines indicate the substituted nucleotides. To obtain a full-length cDNA containing the mutation, secondary PCR was carried out using a mixture of the resulting two fragments described above as a template. Primer 3 and primer 5 were used for secondary PCR. The final PCR product was cloned into pCRII and sequenced. No substitution was found except for the targeted mutation.

Bacterial expression of recombinant protein

Full-length cDNA of wild-type or mutant *TMDP* inserted into pCRII was digested with *Eco*RI and ligated into an *Eco*RI site of pGEX-2T (Pharmacia). *Escherichia coli* DH5α was transformed with pGEX-TMDP (wild-type) or pGEX-TMDP(C138S) (mutant) and cultured overnight at 37 °C in Luria–Bertani medium. The overnight culture was diluted 1:10 in Luria–Bertani medium and incubated at 37 °C for 2 h. Isopropyl β-D-thiogalactoside was then added to the culture at a final concentration of 1 mM and the cells were cultured for induction at 37 °C for 6 h. After the induction, the cells were harvested and suspended in lysis buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/2 mM EDTA/1 % Triton X-100/10 mM $MgCl_2$/10 mM ATP/5 mM benzamidine/1 μg/ml leupeptin/1 μg/ml aprotinin/0.2 mM PMSF/10 mM dithiothreitol] and sonicated for 1 min on ice five times. The lysates were centrifuged at 10000 ***g*** for 20 min at 4 °C. The resultant supernatants were subjected to affinity chromatography using glutathione–Sepharose (Pharmacia). The purified protein was eluted as described by the manufacturer's instructions.

Preparation of substrates for phosphatase assay

For phosphorylation on tyrosyl residues, 100 μg of myelin basic protein (MBP; Sigma) was specifically phosphorylated by 200 units of c-Abl tyrosine kinase (Oncogene Research Products) at 30 °C for 16 h in 50 mM Hepes (pH 7.6)/10 mM $MgCl_2$/2 mM dithiothreitol/0.1 mM sodium vanadate/0.2 mM ATP/150 μCi of [γ-^{32}P]ATP. For phosphorylation on seryl/threonyl residues, MBP was phosphorylated by 0.5 m-unit of protein kinase A (Sigma) at 30 °C for 16 h in 10 mM Tris/HCl (pH 7.0)/5 mM magnesium acetate/0.2 mM ATP/150 μCi of [γ-^{32}P]ATP. There was no phosphorylation of tyrosyl residues. Both phosphorylated substrates were then precipitated by adding trichloroacetic acid to a final concentration of 25 % and centrifuged at 15000 ***g*** for 10 min at 4 °C. The precipitates were washed four times with 25 % trichloroacetic acid, suspended in 2 M Tris base, and dialysed against 50 mM imidazole/HCl (pH 7.0) at 4 °C four times.

Phosphatase assay

Phosphatase assays using *p*-nitrophenyl phosphate (pNPP) as a substrate were performed in 200 μl of assay buffer containing 100 mM sodium acetate (pH 5.0)/1.6 mM dithiothreitol/10 mM pNPP, and enzyme at 30 °C for 10 min. Reactions were terminated by addition of 500 μl of 0.5 M NaOH and the absorbance at 410 nm was measured. Released phosphate amounts were calculated using a millimolar extinction coefficient of 17.8 for the *p*-nitrophenolate ion.

Assays using phosphorylated MBP were performed in 30 μl of assay buffer containing 50 mM imidazole (pH 7.5), 0.1 % β-mercaptoethanol, MBP phosphorylated on tyrosyl (600 nM) or seryl/threonyl (15 μM) residues, and enzyme at 30 °C for 10 min. Reactions were terminated and released ^{32}P was measured as described previously [37].

Hybridization *in situ*

Three 45-mer synthetic oligonucleotides were used as probes for hybridization *in situ*: AS1 and AS2, were antisense to nucleotide residues 86–130 and 547–591, respectively, and S1 was sense to nucleotide residues 86–130. These probes (3 pmol) were labelled with 30 units of recombinant terminal deoxynucleotidyl transferase (Gibco-BRL) in the presence of 50 μCi of adenosine 5′-[α-[^{35}S]thio]triphosphate and purified by passing through a spin column (Princeton Separations).

Fresh frozen sections of the testis from 10-week-old mice were fixed with 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 7.5) for 10 min at room temperature, washed with PBS, treated with 2 mg/ml glycine in PBS, acetylated with 0.1 M triethanolamine containing 0.25% acetic anhydride, and then washed with 2 × SSC/0.1% sarkosyl. The sections were prehybridized in a buffer [50% formamide/30 mM Tris/HCl (pH 7.6)/0.6 M NaCl/0.25% SDS/1 mM EDTA/0.2 mg/ml tRNA/1 × Denhardt's solution] for 1 h at room temperature. After the prehybridization, the slides were washed with 2 × SSC/0.1% sarkosyl, followed by 0.1 × SSC/0.1% sarkosyl and air-dried after ethanol dehydration. The sections were hybridized with ^{35}S-labelled oligonucleotide probes (5×10^5 c.p.m./slide) in 50 μl of prehybridization buffer, supplemented with 0.1 g/ml dextran sulphate and 0.1 M dithiothreitol, for 16 h at 42 °C in a moisture chamber. After hybridization, the slides were rinsed with 2 × SSC/0.1% sarkosyl, washed twice with 0.1 × SSC/0.1% sarkosyl for 40 min at 55 °C, and then dried after ethanol dehydration. Competition experiments were performed simultaneously by adding a 40-fold excess of unlabelled oligonucleotides to the hybridization buffer containing ^{35}S-labelled oligonucleotides. The hybridization signals were visualized by dipping in NTB-2 emulsion (Kodak).

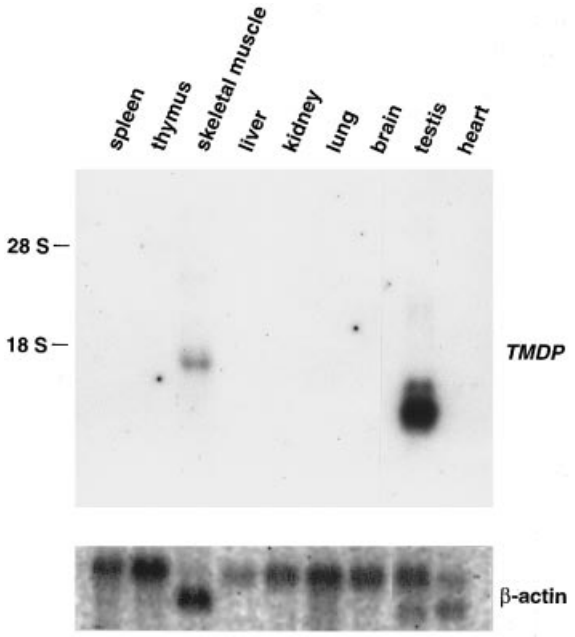


Figure 1 Expression of *TMDP* mRNA in mouse tissues

Total RNA (20 μg) obtained from mouse tissues as indicated was separated on a 1% agarose gel, transferred to a nitrocellulose membrane and hybridized with a random-primed ^{32}P-labelled probe (nucleotides 430–696 in Figure 2b) for *TMDP*. The positions of 28 S and 18 S rRNA are shown on the left. The membrane was reprobed with β-actin as an internal control.

RESULTS

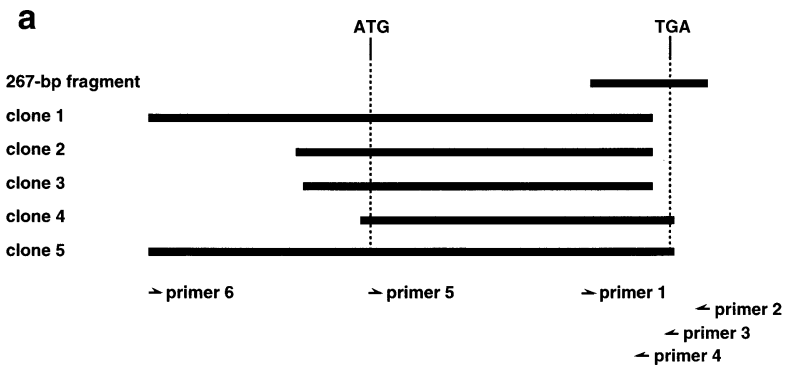
Identification and isolation of a novel DSP

To search for a novel DSP, we screened the dbEST with the nucleotide sequence of human VHR [20] as a probe. The partial sequence of the mouse clone (accession no. AA497918) was found to be novel and to encode a protein related to the DSP family. The deduced amino acid sequence of this clone had the catalytic site motif, VXVHCXAGXSRSXTXXXAYLM, conserved in the DSP family [38]. We cloned a 267-bp cDNA fragment by RT-PCR from the mouse testis based on the sequence of the dbEST clone AA497918. The nucleotide sequence of the 267-bp cDNA fragment was identical to that of the dbEST clone and contained a termination codon following the motif sequence for catalytic domain. Northern-blot analysis showed that this novel DSP is specifically expressed in the testis and skeletal muscle (Figure 1), thus we designated this a novel phosphatase gene, *TMDP*. The TMDP probe detected three species of mRNAs, which were 1.9, 1.7 and 1.6 kb in size. The 1.6- and 1.7-kb mRNAs were specifically and abundantly detected in the testis whereas the 1.9-kb mRNA was specific to skeletal muscle. Then we performed 5′ RACE to isolate a 5′ region of the *TMDP* cDNA containing a methionine initiation codon. As a result of 5′ RACE, three cDNA clones were isolated successfully; clone 1 from mouse skeletal muscle, and clones 2 and 3 from mouse testis (Figure 2a). Interestingly, clones 1, 2 and 3 had identical sequences downstream from nucleotide 322, 146 and 116, respectively, but different sequences at regions upstream from them. Since the methionine codon (nucleotide 324 of clone 1, 148 of clone 2 and 118 of clone 3) was preceded by an in-frame termination codon in each clone, it was thought to be the common translation-initiation site in the three clones. Southern-blot analysis demonstrated that these three clones were derived from a single gene (results not shown). These results indicated that transcription for *TMDP* mRNA was initiated at multiple sites and/or alternative splicing took place just upstream of the initiation codons. We also isolated a full-length *TMDP* cDNA by RT-PCR from the mouse testis (clone 4) and skeletal muscle (clone 5) using specific primers for *TMDP* sequence to verify the sequences of the 5′ RACE products (Figure 2a). The sequence of the entire coding region for *TMDP* was confirmed by clones 4 and 5.

The nucleotide sequences of mouse *TMDP* cDNA and the deduced amino acid sequences are shown in Figure 2(b). The open reading frame of mouse TMDP extends 597-bp and encodes a protein of 198 amino acid residues with a predicted molecular mass of 22.5 kDa. The amino acid sequence of mouse TMDP shows highest similarity with human VHR (45.5% identity; Figure 2c). The amino acid sequences of MKP-1 and CDC25A shows similarity with TMDP protein, with identities of 35.9 and 27.8%, respectively. The N-terminal region of TMDP shows no similarity with other proteins on the database whereas the C-terminal half of TMDP is fairly conserved in other DSPs. Three amino acid residues conserved in all PTPs and thought to be essential for the enzyme activity are also conserved in TMDP at positions Asp-106, Cys-138 and Arg-144.

TMDP has a dual-specificity phosphatase activity

TMDP was predicted as one of DSPs because of the presence of the characteristic active site for the DSP family. To determine whether TMDP possesses an intrinsic phosphatase activity, a recombinant TMDP protein was expressed as a fusion protein with glutathione S-transferase (GST) in *E. coli* and purified as



b

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        10        20        30        40        50        60        70
ATGGACTCGCTACAGAAGCAGGAACTTCGGAGGCCAAAGATTCATGGGGCAGTCCAGGTGTCCCCCTACC
M  D  S  L  Q  K  Q  E  L  R  R  P  K  I  H  G  A  V  Q  V  S  P  Y  Q
        80        90       100       110       120       130       140
AGCCACCCACACTGGCCTCTCTGCAGCGATTGCTGTGGGTCCGTCGGACTGCCACACTGACCCACATCAA
  P  P  T  L  A  S  L  Q  R  L  L  W  V  R  R  T  A  T  L  T  H  I  N
       150       160       170       180       190       200       210
TGAGGTCTGGCCCAACCTTTTCTTGGGAGATGCGTATGCTGCCAGAGACAAGGGTCGTCTAATCCAGCTG
 E  V  W  P  N  L  F  L  G  D  A  Y  A  A  R  D  K  G  R  L  I  Q  L
       220       230       240       250       260       270       280
GGCATTACCCATGTTGTGAATGTGGCTGCGGGCAAGTTCCAGGTGGACACAGGTGCCAAGTTCTACCGTG
G  I  T  H  V  V  N  V  A  A  G  K  F  Q  V  D  T  G  A  K  F  Y  R  G
       290       300       310       320       330       340       350
GAACACCTCTGGAGTACTATGGCATTGAGGCTGATGACAACCCCTTCTTTGACCTCAGCGTCCACTTTCT
  T  P  L  E  Y  Y  G  I  E  A  D  D  N  P  F  F  D  L  S  V  H  F  L
       360       370       380       390       400       410       420
GCCTGTTGCTCGTTACATCAGAGATGCCCTCAATATTCCCCGAAGCCGAGTGCTGGTCCACTGCGCTATG
 P  V  A  R  Y  I  R  D  A  L  N  I  P  R  S  R  V  L  V  H  C  A  M
       430       440       450       460       470       480       490
GGGGTGAGTCGCTCTGCCACAATTGTCTTGGCCTTCCTCATGATCTTCGAGAACATGACACTGGTAGATG
G  V  S  R  S  A  T  I  V  L  A  F  L  M  I  F  E  N  M  T  L  V  D  A
       500       510       520       530       540       550       560
CCATCCAGACGGTGCAGGCCCACCGAGATATCTGTCCCAACTCAGGCTTCCTCCGACAGCTCCAGGTTCT
  I  Q  T  V  Q  A  H  R  D  I  C  P  N  S  G  F  L  R  Q  L  Q  V  L
       570       580       590       600       610       620       630
GGACAACAGGCTGAGGCGGGAAACAGGAAGACTCTGAATTAGGGAGCACCTGAGGCCCTGACCCCTCCAT
 D  N  R  L  R  R  E  T  G  R  L  *
       640       650       660       670       680       690
CCAGCGTGGCCTAACCTGACCTACCTGGCCCTGGGAATGGTAGCCTCTGCTGTTTCCAGAGATCCT
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c

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TMDP    1  MDSLQKQELRRPKIHGAVQVSPYQPPTLASLQRLLWVRRTATLTHINEVWENLFLGDAYA
VHR     1                  MSGSFELSVQDLNDLLSDGSGCYSLPSQPCNEVTERIYVGNASV

TMDP   61  ARDKGRLIQLGITHVVNVAAGK-F-QVDTGAKFYRGTPLEYYGIEADDNPFFDLSVHFLP
VHR    45  AQDIPKLQKLGITHVLNAAEGRSFMHVNINANFYKDSGITYLGIKANDTQEFNLSAYFER

TMDP  119  VARYLRDALNIPRSRVLVHCAMGVSRSATIVLAFLMIFENMTLVDAIQTVQAHRDICPNS
VHR   105  AADFIDQALAQKNGRVLVHCREGYSRSPTLVIAYLMMRQKMDVKSALSIVRQNREIGPND

TMDP  179  GFLRQLQVLDNRLRRETGRL
VHR   165  GFLAQLCQLNDRLAKEGKLKP
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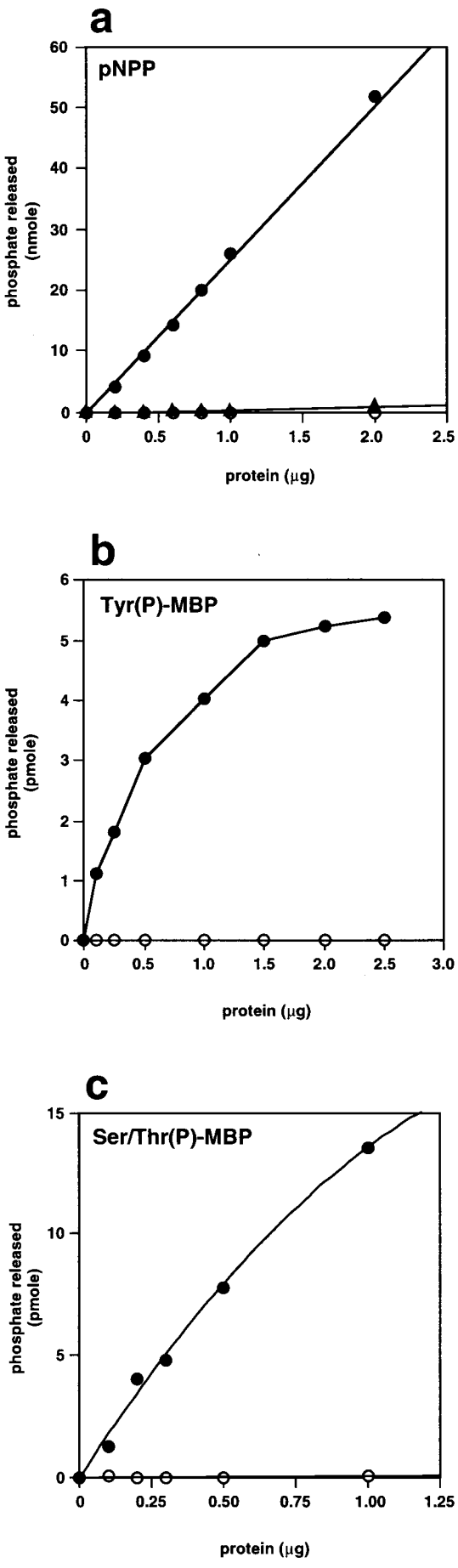
Figure 2 cDNA clones, nucleotide sequence and deduced amino acid sequence of TMDP

(**a**) Scheme of cDNA clones obtained by RT-PCR and 5′ RACE is shown. The 267-bp fragment and clones 2–4 were from mouse testis, and clones 1 and 5 were from mouse skeletal muscle. The positions and orientations of primers 1–6 are also shown. (**b**) The nucleotide sequence covering the entire coding region and the deduced amino acid sequence of mouse *TMDP* are shown. The termination codon is marked by the asterisk. The conserved active-site motif for DSPs is boxed. (**c**) The alignment of amino acid sequences of mouse TMDP and human VHR is shown. Identical amino acid residues are indicated by shaded boxes. Gaps are shown by dashes.



described in the Materials and methods section. The recombinant protein was subjected to a phosphatase assay using pNPP, tyrosine-phosphorylated MBP [Tyr(P)-MBP] and serine/threonine-phosphorylated MBP [Ser/Thr(P)-MBP] as substrates. The recombinant TMDP dephosphorylated pNPP, depending on the amounts of enzyme added (Figure 3a). GST alone could not dephosphorylate pNPP (results not shown). Sodium vanadate (1 mM), a specific inhibitor for PTP, completely inhibited the phosphatase activity of TMDP. Cys-138 in the active-site motif of TMDP is expected to be an essential residue for phosphatase activity, as in other tyrosine phosphatases. Thus we analysed the effects of the cysteine residue on phosphatase activity. A recombinant TMDP(C138S) protein, which had Cys-138 changed to Ser, displayed no detectable pNPP phosphatase activity with up to 2 μg of protein (Figure 3a). TMDP also dephosphorylated Tyr(P)-MBP, depending on the amounts of enzyme added, up to 1.5 μg of protein (Figure 3b). The phosphatase activity of TMDP towards Ser/Thr(P)-MBP was also examined. The recombinant TMDP protein exhibited significant activity towards Ser/Thr(P)-MBP, depending on the amount of enzyme added (Figure 3c). The specific activity of TMDP towards Ser/Thr(P)-MBP was 1.59 pmol/min per μg, which is similar to that (1.12 pmol/min per μg) towards Tyr(P)-MBP up to 0.25 μg of enzyme added. The reactions were linear under these conditions. The TMDP(C138S) protein could dephosphorylate neither Tyr(P)-MBP nor Ser/Thr(P)-MBP (Figures 3b and 3c). Okadaic acid (1 μM) or 40 nM microcystin LR, inhibitors for protein phosphatases 1 and 2A, did not affect the activity of TMDP towards Tyr(P)-MBP or Ser/Thr(P)-MBP, whereas 1 mM sodium vanadate completely abolished the activity of TMDP towards both Tyr(P)-MBP and Ser/Thr(P)-MBP (results not shown). These data demonstrate that TMDP has a phosphatase activity towards both phospho-seryl/threonyl and -tyrosyl residues and suggest that Cys-138 of TMDP is essential for the catalytic activity towards both phospho-seryl/threonyl and -tyrosyl residues of substrates.

Expression of *TMDP* mRNA is developmentally regulated during spermatogenesis

The *TMDP* mRNA was abundantly expressed in the testis (Figure 1). The time course of *TMDP* expression in the mouse testis after birth was examined to determine the involvement of *TMDP* in the meiotic process. The *TMDP* mRNA was not detected in the testis up to 2 weeks after birth but first appeared at 3 weeks, gradually increased, and reached a plateau at 7 weeks (Figure 4a). To elucidate the relationship between the TMDP expression and spermatogenesis, testicular cells were separated into spermatocytes, round spermatids and elongated spermatids from 10-week-old male mice, and the total RNAs extracted from each fraction were subjected to Northern-blot analysis. The purities of spermatocytes, round spermatids and elongated spermatids were about 80, 80 and 75 %, respectively. The *TMDP* mRNA was highly expressed in the fraction of spermatocytes and round spermatids (Figure 4b). Considering the low purity of

Figure 3 Phosphatase activity of recombinant TMDP and the mutant TMDP(C138S)

The phosphatase activities of both GST-TMDP (●) and GST-TMDP(C138S) (○) were determined using pNPP and MBP as substrates at 30 °C for 10 min. (**a**) Dose-dependent dephosphorylation of 10 mM pNPP by increasing amounts of GST-TMDP and GST-TMDP(C138S). The activity of GST-TMDP in the presence of 1 mM sodium vanadate was also shown (▲). (**b** and **c**) Dose-dependent dephosphorylation of (**b**) 0.6 μM Tyr(P)-MBP and (**c**) 15 μM Ser/Thr(P)-MBP by increasing amounts of GST-TMDP and GST-TMDP(C138S). The data shown are the averages of duplicated experiments.

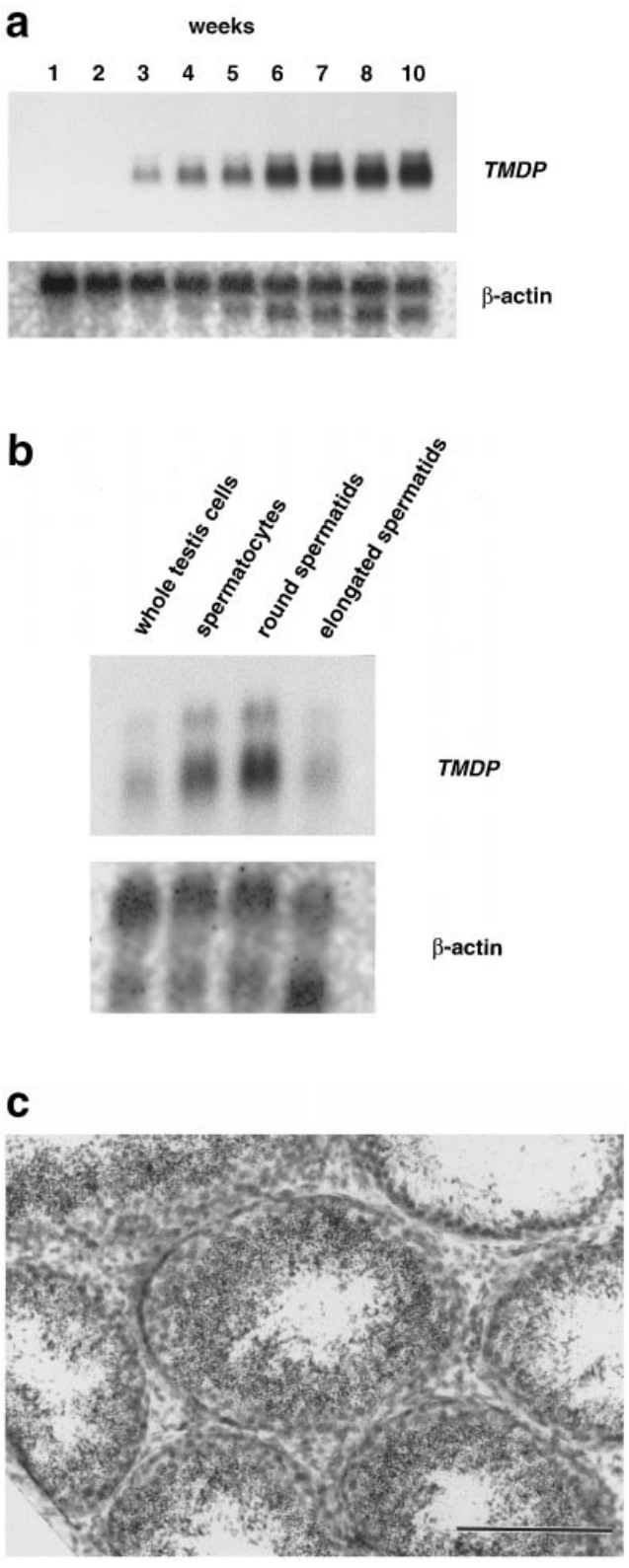


Figure 4 Expression of *TMDP* mRNA in the testes

Total RNA (20 μg) was isolated from the testes of mice at the ages indicated (**a**) or from fractionated spermatogenic cells (**b**). Northern-blot analysis was performed as described in Figure 1. The membrane was reprobed with β-actin as an internal control. (**c**) Hybridization *in situ* for *TMDP* mRNA in the testis is shown. Sections of the 10-week-old male mouse testis were hybridized with the ^{35}S-labelled antisense oligonucleotide probe AS1. A bright-field microscopic image with counterstaining by haematoxylin is shown. Scale bar, 100 μm.

each fraction, the detectable expression in the fraction of elongated spermatids was thought to be due to a contamination of round spermatids or spermatocytes.

To investigate further whether *TMDP* is specifically expressed in restricted testicular germ cells and whether *TMDP* expression is regulated during spermatogenesis, *TMDP* mRNA in the testis was examined by hybridization *in situ*. Frozen sections of the 10-week-old mouse testis were hybridized with ^{35}S-labelled *TMDP* antisense probes, AS1 (Figure 4c) and AS2 (results not shown). By bright-field microscopy for emulsion-dipped sections, silver grains representing *TMDP* mRNA were restricted in the middle zone between the centres and the basal lamina of seminiferous tubules (Figure 4c), where late spermatocytes and early round spermatids are enriched. The *TMDP* mRNA was not detected in Leydig cells, spermatogonia or spermatozoa. No significant signal was detected in hybridization using sense probe S1 or antisense probe AS1 in the presence of a 40-fold excess of unlabelled AS1 oligonucleotide (results not shown). These results indicate that the expression of *TMDP* in the testis is restricted to testicular germ cells, particularly in spermatocytes and round spermatids.

DISCUSSION

In this study, we identified and characterized a novel DSP, named TMDP. Although the molecular mass of TMDP is similar to that of VHR, the similarity between TMDP and VHR as well as between TMDP and MKP or CDC25 is only observed in the catalytic region. Thus TMDP is thought to be a novel DSP categorized as a new low-molecular-mass type of DSP. The N-terminal region of TMDP shows no similarity with other DSPs, suggesting that this region may contribute to the substrate recognition or the subcellular localization of TMDP.

The specific activities of TMDP towards Tyr(P)-MBP and Ser/Thr(P)-MBP were similar under present conditions, although we could not determine precisely the specific activities from the initial rates because of the limitation of phosphorylation on Tyr(P)-MBP. Previous results of specific activities toward phospho-tyrosyl and phospho-seryl/threonyl substrates are variable depending on DSP molecules. For example, VHR catalysed hydrolysis of tyrosine-phosphorylated Raytide more than 300 times faster than hydrolysis of serine-phosphorylated Kemptide or other serine/threonine-phosphorylated peptide substrates [39]. BVP, a baculoviral phosphatase, dephosphorylated tyrosine-phosphorylated substrates more rapidly than serine/threonine-phosphorylated substrates [40]. On the other hand, VH1, a DSP encoded by *Vaccinia* virus, dephosphorylated serine-phosphorylated casein seven times more rapidly than tyrosine-phosphorylated Raytide [3]. Further experiments for determination of kinetic parameters of phospho-tyrosine and phospho-serine/threonine dephosphorylation are required for precise determination of the substrate specificity of DSPs including TMDP.

The *TMDP* mRNAs of 1.9 kb and the others of 1.7 and 1.6 kb are expressed exclusively in skeletal muscle and testis, respectively. In general, mRNA variants with different sizes are generated by several mechanisms, such as alternative promoter usage, splicing and polyadenylation. Although we did not isolate full-length cDNAs corresponding to the three transcripts, we could isolate three clones which had different sequences in the 5′-untranslated regions. It is a possibility that the size differences of *TMDP* mRNAs is generated by alternative promoter usage and/or exon skipping. Maiti et al. [41] has reported that the proximal promoter of the homeobox gene *Pem* is active only in the testis whereas the distal promoter is active in placenta, ovary,

and to a lesser extent in skeletal muscle, although encoded proteins are identical [41]. The c-*mos*, c-*abl*, *pim-1* and *GATA-1* genes have also been reported to use alternative promoters in the germ cells of the testis that differ from the promoters used in somatic cells [42–44].

Several protein phosphatases are reported to be expressed specifically in the testis [25–28,45–47]. Serine/threonine protein phosphatase PP1γ2 is abundant in the rat testis and localized in the nuclei of late spermatocytes and early spermatids [29]. Target disruption of the *PP1*γ gene impaired spermiogenesis [35]. PTP testis-specific tyrosine phosphatase (Typ) is also expressed exclusively in testicular germ cells that undergo meiosis [30]. These findings suggest that these protein phosphatases are involved in the stage-specific regulation of spermatogenesis. TMDP is the first DSP identified as being expressed mainly during meiosis.

Recently, it has been reported that several protein kinases are expressed abundantly in the testis. The protein tyrosine kinases, FerT [31], TSK-1 [32], c-Kit [48], Sp42 [33] and c-Abl [49], are also suggested to be involved in spermatogenesis. Interestingly, Nek1, a dual-specificity kinase, is most abundantly expressed in the testis and the levels are much higher in spermatocytes and round haploid spermatids [34]. The expression patterns of *nek1* mRNA by hybridization *in situ* is almost paralleled with those of *TMDP*. TMDP may function during spermatogenesis through regulation of phospho-tyrosine and -serine/threonine contents of target proteins by co-operating with Nek1.

In summary, TMDP, a novel DSP, is abundantly expressed in the testis and the expression is up-regulated at the meiotic stage during spermatogenesis, suggesting an important role of TMDP during spermatogenesis. The identification of the novel dual protein phosphatase suggests existence of an unknown signalling pathway including phosphorylation of both serine/threonine and tyrosine residues in spermatogenesis.

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