Molecular cloning of haploid germ cell-specific tektin cDNA and analysis of the protein in mouse testis

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Abstract Tektins are a class of proteins that form filamentous polymers in the walls of ciliary and flagellar microtubules. We report here the molecular cloning of a new member of the tektin family, tektin-t, identified from a mouse haploid germ cell-specific cDNA library. Tektin-t mRNA encodes a protein of 430 deduced amino acids possessing RSNVELCRD, the conserved sequence of tektin family proteins. Western blotting showed a single band having a molecular weight of 86 kDa in the mouse testis. Immunohistochemistry of the testis showed that tektin-t is localized in the flagella of elongating spermatids from developmental step 15 to maturity.

Key words: Mouse; Testis; Tektin; cDNA library; Sperm

1. Introduction

In the testis, germ cell development continues throughout the adult life. This complex process, referred to as spermatogenesis, consists of three events: the proliferation and differentiation of spermatogonia, the meiotic prophase of spermatocytes and the morphological change of post-meiotic haploid spermatids to sperm [1]. Mouse germ cell differentiation from spermatogonial stem cells to sperm is completed in seminiferous tubules in approximately 1 month under the complex regulation of many different molecules, including hormones and growth factors. In the mouse testis, spermatogonia differentiate to spermatocytes at 8 days after birth. The differentiation of haploid germ cells starts at 18 days. After that, haploid germ cell-specific events such as assembly of the flagellum, condensation of the nucleus and formation of the acrosome to make highly differentiated active sperm occur. Recently, several kinds of haploid germ cell-specific genes have been isolated and characterized [2–4]. However, few of the genes encoding the structural proteins of sperm have been cloned.

The development of the sperm flagella is a major specific event in spermiogenesis. The structure of the flagella has been examined by electronmicroscopy [5,6] and a few structural genes of flagella were cloned [7–9]. Although morphologically, the sperm flagella differ a little in each species, the primary structural protein should be similar. Tektins are components of flagella microtubules and most of the characterization has been done on sperm flagella of sea urchin [10]. Immunological evidence suggested that tektins or tektin-like proteins were present in stable microtubular organelles [11].

To study spermiogenesis, we have cloned many genes specifically expressed in haploid germ cells from the subtracted cDNA library [2], which was generated by subtracting mRNAs of 17 days old mouse testes from cDNAs of 35 days old mouse testes. Here, we report the isolation and molecular analyses of tektin-t, a cDNA encoded tektin-like protein specifically expressed in haploid germ cells of the mouse testis.

2. Materials and methods

2.1. Preparation of the cDNA library

Total RNAs were extracted by the guanidine thiocyanate/CsTFA method [12] from testes of C57BL/6 mice followed by the purification of poly(A)+ RNAs. A cDNA library was prepared as described by Gubler and Hoffmann [13] with some modifications [14,15]. Briefly, cDNAs were synthesized in a reaction mixture including 5Me-dCTP with reverse transcriptase (Superscript II) and 1.6 μg of oligo (dT) primer carrying a *Not*I site from 7 μg of mouse testis poly(A)+ RNAs. The reaction mixture was treated with RNAse H, followed by reaction with DNA polymerase I. Each end was blunted with T4 DNA polymerase and ligated to an unphosphorylated *Bgl*II-*Sma*I adapter. After digestion with *Not*I, a CROMA spin-400 column (Clontech, CA, USA) removed DNA fragments of less than 300 bp. The resulting cDNA fragments longer than 300 bp were directionally inserted between *Not*I (dephosphorylated) and *Bgl*II sites of the pAP3*neo* vector (Takara, Japan). The ligation mixture was electroporated into MC1061A cells as described [16]. The complexity of the cDNA library used here was 4.0×10^6 colony forming units (cfu).

2.2. Screening and DNA sequencing

A haploid germ cell-specific cDNA library in the pAP3*neo* vector (Takara, Japan) was generated by subtracting mRNAs of 17 days old testes from a cDNA library of 35 days old testes [2]. Plasmid DNA of each clone randomly picked from the subtracted cDNA library was screened for haploid germ cell-specific expression by Northern blot analysis using mRNAs of the testes taken from 17 and 35 days old mice [2]. We named these haploid germ cell-specific sequence tags: transcription increased in spermiogenesis (TISP). One of them, TISP76, showed significant homology with tektin mRNA in the GenBank, EMBL and DDBJ databases. Then, the complete cDNA of TISP76 was obtained by screening the cDNA library of adult testes. The adult testicular cDNA library of *Escherichia coli* MC1061A cells was diluted to seed at 2×10^5 cfu on a nitrocellulose filter placed on a LB plate. After incubation at 37°C, grown colonies were transferred to two nylon replica filters and then, the filters were sequentially soaked in the following solutions at room temperature: 5 min in 0.5 N NaOH+1.5 M NaCl, 5 min in 0.5 M Tris-HCl (pH 7.4)+ 1.5 M NaCl and 5 min in 2×SSC. After baking at 80°C for 2 h, the filters were washed and bacterial debris was removed. A [α-^{32}P]dCTP-labelled probe was prepared by a BcaBest random primer kit (Takara, Japan) using a 1.3 kb *Eco*RI-*Not*I fragment of the partial tektin-t cDNA fragment. Then, the filters were hybridized with the probe at 65°C for 20 h (4×SSC, 10×Denhardt's solution, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA).

Dideoxy chain termination sequencing reactions [17] were performed with fluorescent dye-labelled primers and thermal cycle se-

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(A)

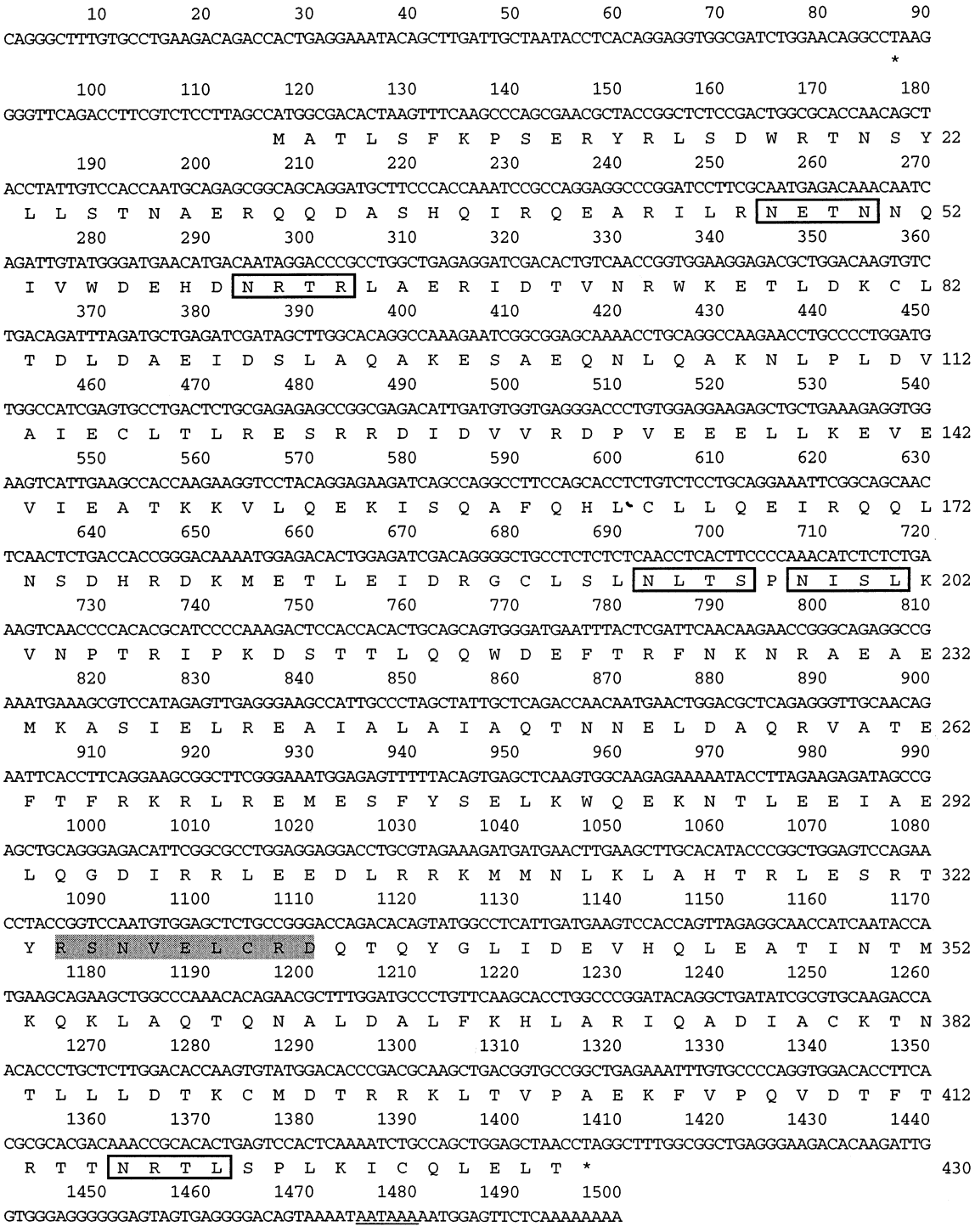

Fig. 1. Sequence analysis of tektin-t. (A) The composite nucleotide sequence and the deduced amino acid sequence of tektin-t isolated from a haploid germ cell-specific cDNA library of the mouse testis. Asterisks are positioned to stop codons (nt. 87–89 and 1407–1409). A consensus polyadenylation sequence is underlined. Shadowed letters indicate the amino acid sequence corresponding to the RPNVELCRD repeat conserved among tektin gene family proteins. Potential *N*-glycosylation sites are boxed. (B) Multiple alignment of the deduced amino acid sequence of tektin-t with tektins A1, B1 and C1 from sea urchin sperm flagella. Gaps have been inserted to maximize the matching. Shadowed letters denote identical amino acid residues, conserved in at least three of them. Asterisks indicate the conserved RPNVELCRD repeat.

(B)

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Tektin-t    6:FKPSERYRLSDWRTNSYLLSTNAERQQDASHQIRQEARILRNETNNQIVWDEHDNRTRLA  65
tektin A1  61:FR-SGKHTTQEWHESNYNKYFQSFTDRDNAERLCHESKQLSNEEHALTMRTQADVTKKLG 119
tektin B1   1:------------------MLR-NS--VITPTPPAKNLASFVTKPE-TDIM-GYHDNTTRLS  38
tektin C1  11:F-T---H-G-EWNYSNHANYNSAEKQRASAERLIDESDRLIDEEDEATKKTQRDVNKKFE  64

Tektin-t   66:ERIDTVNRWKETLDKCLTDLDAEIDSLAQAKESAEQNLQAKNLPLDVAIECLTLRESRRD 125
tektin A1 120:DRMNDINFWKFELNREIEEMIEETDLLCAQKKRLENALDATEVPLKIARDNLTCRSRRQD 179
tektin B1  39:NRIDDIETWRETLEKTLADVDEEIRKLEEDKDLAERALEAKALPLDVASECKTLRDGRRD  98
tektin C1  65:QRLDDVTYWKDELDRKLKDSKDEIEMLLAYKTRLENALEACREPLAIVNQCLNDREGRIG 124

Tektin-t  126:IDVVRDPVEEELLKEVEVIEATKKVLQEKISQAFQHLCLLQEIRQQLNSDHRDKMETLEI 185
tektin A1 180:IDLVGDRVEMALNKEVDIITKVQDLLKRTLEQSDRQIKLNRGSKHKLTMDWSDKLSAFKI 239
tektin B1  99:NDVVDDLANSEVGKEIDVIEGIKDALQAKVSSAFEQLCLLQEARQQLHADLRDKTEAKKI 158
tektin C1 125:IDLVHDDVEKNLLKEREVIMGVMALLQKTLDQVIEQIRIMRSRRYNLEKDLTDKFGALDI 184

Tektin-t  186:DRGCLSLNLTSPNISLKVNPTRIPKDSTTLQQWDEFTRFNKNRAEAEMKASIELREAIAL 245
tektin A1 240:DEKCTGLNNNSTEIQYKEGSAKFEAVQTNPQSWAEFSHDNVVRAEHERLASQQLRNLIDQ 299
tektin B1 159:DTYCHDLTISSPDICYQPNSTRTPKGSTTPQTWEDFSRYNKDRADAEMRSSQRLREAIHS 218
tektin C1 185:DQDCRNLRDDNSTLQFKGGVAKIETNSVTPEDWQSFSNENILKAERDRKNSADLRAVVDS 244

Tektin-t  246:AIAQTNNELDAQRVATEFTFRKRLREMESFYSELKWQEKNTLEEIAELQGDIRRLEEDLR 305
tektin A1 300:ILTDTSNDMREQCNTVNTEFARRIEEMNDAKTKMENHLLKTVEDIAGMEKNIKDLTQAVK 359
tektin B1 219:TVAQTDNDLEAQRQATEFALRKRIHEMKRAKDEDEWQKKNTEEEIAKQERNIRELEQAIK 278
tektin C1 245:LLKTTADDMQQQVDDTNLAFSKRIRETDMTKGKLETHLAKVEGQMREMEENIQKLQKGVD 304

Tektin-t  306:RKMMNLKLAHTRLESRTYRSNVELCRDQTQYGLIDEVHQLEATINTMKQKLAQTQNALDA 365
tektin A1 360:DKEAPMKVAQTRLDHRTHRPNVELCRDPAQYRMVQEVGEIQDSIDKLQQKLAESKASLKD 419
tektin B1 279:DKENPLKLAMTRLENRTYRPNVELCRDNAQYGLVNEVHEIQDSIKALEKKLQDAHNARDA 338
tektin C1 305:DKMGPKKLSETRLDARTNRPNVELCRDPVQYRLIGEVTEINTSIARLQATLAQAQSELKG 364
                                 *********

Tektin-t  366:LFKHLARIQADIACKTNTLLLD-TKCMDTRRKLTV-PAEKFVP                406
tektin A1 420:LMDTRMALEKEIALKKNSIFVDRDKCLKFRTRYPSTSKLVGYQ                462
tektin B1 339:CEKQLYRINKDLELKNNSLDLD-NKCMQVREKLTTGPVTQTMN                395
tektin C1 365:LIRNQLNLQEDIDIKSQSLNVDDTQCMTLR-R-SINIK-R-Y-                402
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Fig. 1 (continued)

quencing kits purchased from Li-COR. The reaction products were analyzed with a Model 4000 (Li-COR, NE, USA). Three independent positive clones, isolated by screening 2×10^5 colonies, were sequenced. Then, the GenBank, EMBL, DDBJ, Swiss-Prot and PIR databases were searched for homology with the cDNA or amino acid sequence of this clone.

2.3. Northern blot analyses

Total RNAs were isolated from various organs of mice (C57BL/6 strain) with RNA zolTM B (Tel-test, CA, USA). Germ and other somatic cells of the testes were prepared as described in our previous report [18]. Total RNAs were extracted according to the manufacturer's recommendation and quantified by optical density measurement. 10 μg of mRNA containing 2.2 M formaldehyde was separated on a 1.0% agarose gel with 0.66 M formaldehyde and transferred to a nitrocellulose filter [19]. Hybridization was performed with probes of partial tektin-t cDNA and the mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a control. The filter was pre-hybridized for 3 h and then hybridized for 20 h in a solution of 5×SSC, 5×Denhardt's solution, 0.1% SDS and 100 μg/ml of sheared salmon sperm DNA at 42°C with an [α-^{32}P]dCTP-labelled cDNA probe. Each filter was washed at 60°C in 0.2×SSC buffer containing 0.1% SDS. Signals were detected with an Image analyzer (Fuji Film, Japan).

2.4. Anti-tektin-t antiserum

The *Bam*HI-*Hin*dIII fragment (0.8 kb) of tektin-t cDNA corresponding to amino acid residues 44–313 was subcloned into the pET30c expression vector (Novagen, WI, USA). The protein histidine-tagged at the N-terminus was expressed in *E. coli* BL21 by induction with IPTG, purified with Ni^+ Spin kit (Qiagen, Germany) according to the manufacturer's protocol and then used to raise polyclonal antiserum in rabbits together with GERBU Adjuvant 100 (GERBU Biotechnik, Germany).

2.5. Western blot analyses

Protein samples of various mouse tissues (C57BL/6 strain) were prepared with RIPA buffer (10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and 1 mM PMSF). After centrifugation, aliquots of the samples (50 μg/lane) were separated by 10% SDS-PAGE and transferred to PVDF filters (Millipore, MA, USA), followed by blocking with TBS-T (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM KCl, 0.05% Tween 20) containing 5% dried milk. The filters were incubated

in TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM KCl) containing diluted antibodies (×1500), treated with horseradish peroxidase-conjugated anti-rabbit IgG and developed with the POD staining kit (Wako, Japan).

2.6. Immunohistochemistry

Frozen sections (10 μm) of the testis were fixed with 4% PLP solution (containing 4% paraformaldehyde, 75 mM L-lysine monohydrochloride, 25 mM NaPi and 10 mM $NaIO_4$) for 20 min at 4°C and then reacted with diluted polyclonal anti-tektin-t antiserum (×50) after blocking with 5% normal sheep serum. The sections were treated with 0.3% H_2O_2 in methanol at room temperature for 30 min and reacted with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Japan), followed by diaminobenzidine and then counter-stained with hematoxylin. The sections were examined under a microscope. Mature sperms were taken from the epididymis of an adult mouse and were spotted on Micro Slide Glass (Matsunami Glass, Japan). Sperm samples were fixed with 4% PLP solution for 30 min at 4°C and reacted with diluted polyclonal anti-tektin-t antiserum (×50) after blocking with 5% normal goat serum. Then, the samples were treated with FITC-labelled anti-rabbit Igs antibody (Amersham Pharmacia Biotech, Japan) and examined under a fluorescent microscope.

3. Results

3.1. Cloning and sequence analysis of tektin-t cDNA

We have isolated a haploid germ cell-specific cDNA clone (TISP76) from a subtracted cDNA library of mouse testes. For the isolation of a full-length clone, an adult mouse testicular cDNA library of pAP3*neo* (Takara, Japan) was screened with the partial cDNA clone from the subtracted cDNA library and 15 independent clones were isolated. All of the inserts of newly isolated cDNA clones were similar in size, approximately 1.5 kb. Sequence analysis of all the clones revealed the same long open reading frame of 1290 nucleotides (nt) (nt 117–1406) encoding a predicted 430 amino acid residues (Fig. 1A) (GenBank/EMBL/DDBJ accession number AB027138). A presumed polyadenylation signal, AATAAA, was located at nucleotide positions 1475–1480. The poly(A) tail started at position 1493. A computer-assisted database search indicated that the deduced amino acid sequence of this clone showed some homology with sea urchin other tektins (~32%) and the highest homology (52% identity) with B1 (Fig. 1B). So, we named this gene tektin-t. The deduced amino acid sequence of tektin-t possessed a specific nona-peptide, RSNVELCRD at 324–332 (Fig. 1A), corresponding to the RPNVELCRD repeat conserved among tektins [10].

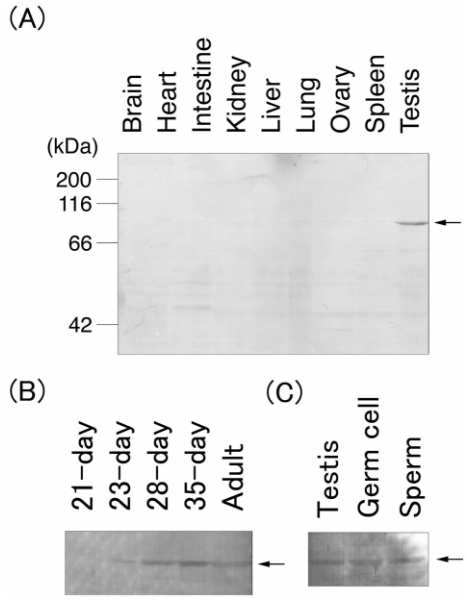

Fig. 3. Western blot analysis of the tektin-t protein. Protein samples (50 μg/lane) prepared from various organs of adult mouse testes of various ages and fractionated cells of the testis were examined with antiserum raised in rabbit against recombinant tektin-t. (A) Organ-specific expression of tektin-t. (B) Specific expression of tektin-t in male germ cell development. Proteins were extracted from the mouse testes at the age of 21, 23, 28 and 35 days and more than 2 months as the adult. (C) Expression of tektin-t in fractionated testicular germ cells and epididymal sperm of mouse. The position of molecular weight markers (kDa) is indicated at the left margin.

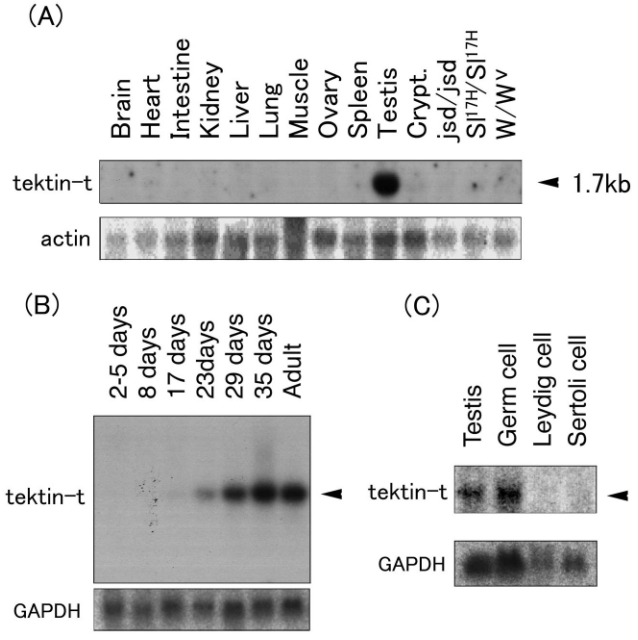

Fig. 2. Northern blotting of tektin-t. (A) Organ-specific expression of tektin-t mRNA. RNAs from various organs of mouse and from the cryptorchid (Crypt.) and mutant (jsd/jsd, Sl^{17H}/Sl^{17H}, w/w^v) mouse testes containing no differentiated germ cells were examined. (B) Specific expression of tektin-t mRNA in male germ cell development. RNA samples from 2–5, 8, 17, 23, 29 and 35 days old and the adult testes were examined. (C) Specific expression of tektin-t mRNA in fractionated cells of the mouse testis. RNA samples from germ cell, Leydig cell and Sertoli cell fractions were examined. Filters were re-hybridized with mouse actins (A) and mouse GAPDH cDNA (B and C) as a control (see lower panels). The tektin-t transcript at 1.7 kb is indicated by an arrowhead.

3.2. Analyses of tektin-t mRNA by Northern blotting

A tektin-t mRNA transcript of about 1.7 kb was detected in the wild mouse testis but not in the testes from cryptorchid and mutant (jsd/jsd, Sl^{17H}/Sl^{17H} and w/w^v) mice by Northern blot analysis (Fig. 2A). The mutant mice testes contain no differentiated germ cells [14,20–22]. To investigate the developmental change of transcription in the mouse testis, total testicular RNAs at the age of 2–5, 8, 17, 23, 29 and 35 days were analyzed. The tektin-t mRNA transcript was slightly detected at the age of 17 days and significant signals were detected from the age of 23 days. More than 23 days old, the expression level was increased with age (Fig. 2B). Furthermore, it was expressed in testicular germ cells but not in somatic cells such as Sertoli or Leydig cells (Fig. 2C).

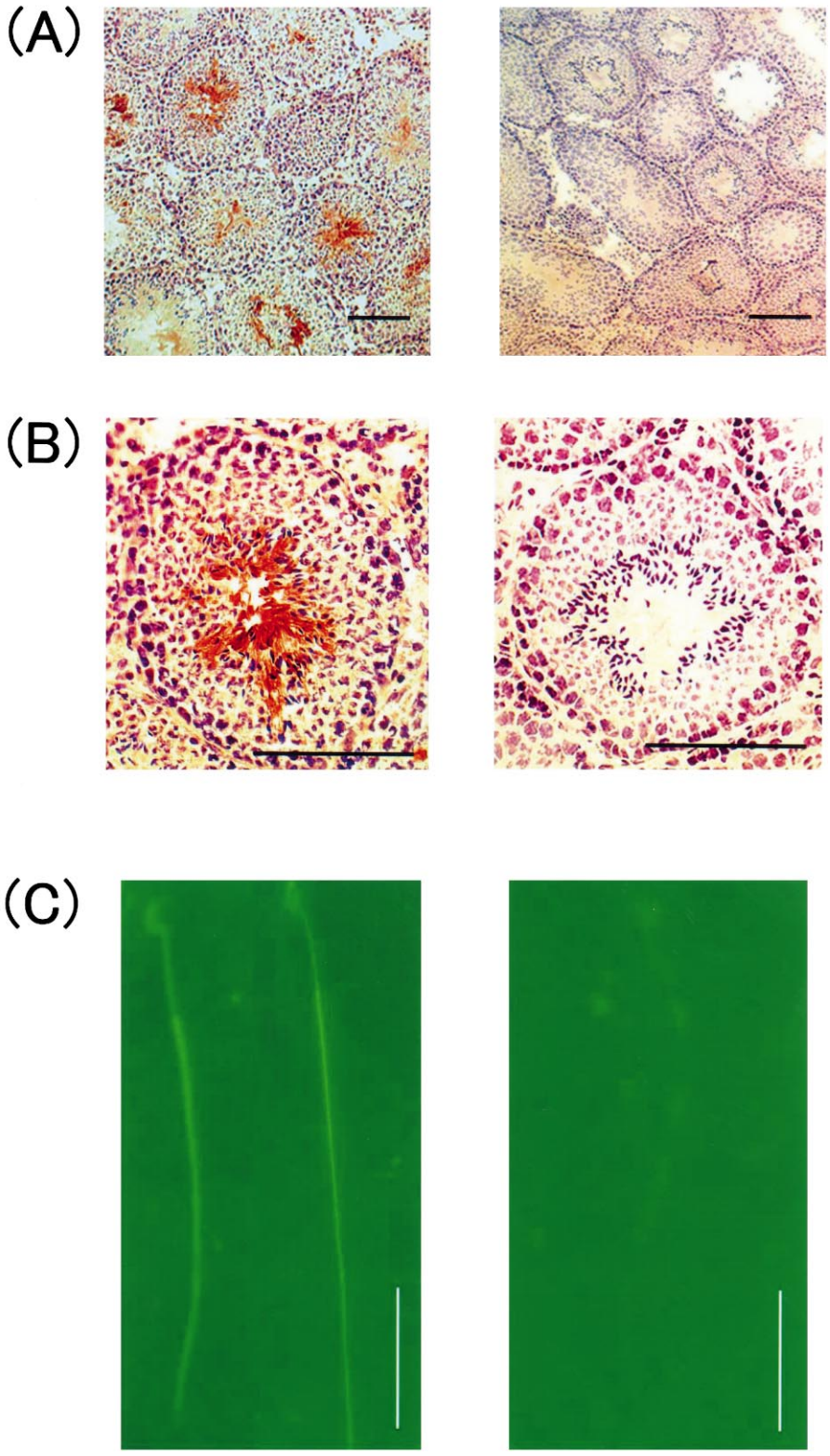

Fig. 4. Immunohistochemical analysis of tektin-t. The flagella of elongating spermatids at step 15 to maturity were selectively stained in mouse testis with the anti-tektin-t antiserum (left side) and pre-immune serum as a control (right side). Low- (A) and high- (B) magnification micrographs. Sections were counterstained with hematoxylin. Scale bar = 100 μm. (C) Tektin-t was localized to the flagella of mature mouse sperm (left) and no staining was detectable in the right side control. Original magnification is ×400. Scale bar = 10 μm.

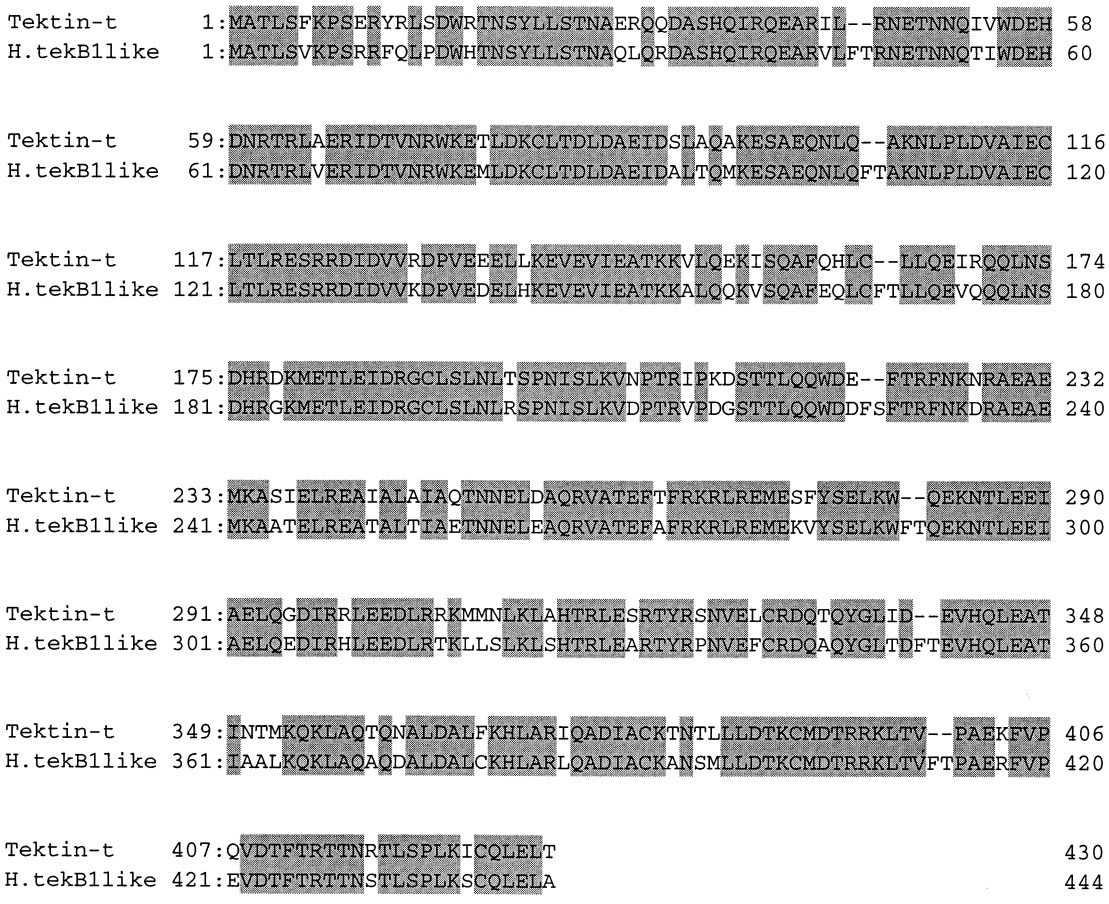

Fig. 5. The deduced amino acid sequence alignment of tektin-t with human tektin B1-like protein. Gaps are to maximize the alignment. Shadowed letters show identical amino acid residues.

3.3. Western blot analysis and immunohistochemistry of tektin-t protein

Rabbit polyclonal antiserum was raised against a histidine-tagged recombinant tektin-t protein isolated from *E. coli*. Western blot analysis of the protein extracts from various mouse organs showed that tektin-t having a molecular weight of 85 kDa was exclusively expressed in the testis (Fig. 3A). During male germ cell development, tektin-t was detected first at 23 days of age (Fig. 3B). Immunoblot analysis of the protein extracted from fractionated cells of the testis showed that a positive signal was detected in germ cells including sperm (Fig. 3C).

Immunohistochemical staining of the testicular cross-sections showed the expression of tektin-t at the late stages of spermiogenesis, from elongating spermatids at developmental step 15 to mature sperms at the flagella (Fig. 4A and B). Furthermore, we used the mature sperm from adult mouse epididymis to investigate the locus of tektin-t in more detail and found that the localization of tektin-t was restricted to the flagella (Fig. 4C).

4. Discussion

Tektins were originally identified as a set of structural proteins of sea urchin flagellar microtubules and most of the structural chemistry has been done in sea urchin sperm [23,24]. Among the sequences for tektins A1, B1 and C1 (GenBank/EMBL/DDBJ accession numbers M97188, L21838 and U38523, respectively) from sea urchin (*Strongylocentrotous purpuratus*) sperm flagellar microtubules, the predicted amino acid sequence of tektin-t showed the highest homology with tektin B1. The amino acid identity is 52%, while with other tektins, it is approximately 32%. Tektin-t should be the mouse germ cell-specific counterpart of tektin B1 reported previously. Furthermore, the tektin-t showed a high homology with the deduced amino acid sequence of human tektin B1-like protein (GenBank/EMBL/DDBJ accession number AF054910) (Fig. 5), suggesting that human tektin B1-like protein registered in databases might be the counterpart of tektin-t. The nona-peptide sequence RPNVELCRD, or its variants, may be an important domain to function [25]. Tektin-t also possessed an amino acid sequence, RSNVELCRD, considered to be a variant of the conserved repeat of tektins. Tektin-t has five predicted *N*-glycosylation sites at amino acid residues 47–50, 60–63, 193–196, 198–201 and 416–419. Although the calculated mass of tektin-t was about 50 kDa, anti-tektin-t antiserum detected a protein of 85 kDa in mouse testis. *N*-glycosylation may be the cause for the delay on SDS-PAGE.

Recently, murine partial cDNA similar to tektin A1 [26], the human homologue of tektin B1 and the murine homologue of tektin C1 [25] were isolated from a cDNA library of mammalian testes. Although detailed analyses of these three cDNAs were not reported, at least two of them were strongly expressed in the testis. Tektins form the unique pro-

tofilament of flagellar microtubules by direct interaction with a tubulin filament [25,27]. Highly expressed testicular tektins should contribute to the formation of sperm flagella in adult testis. In fact, we showed here that tektin-t protein was localized specifically in the flagella from the elongating spermatid to the mature sperm stage, suggesting that tektin-t plays important roles in the formation of sperm flagella and in sperm motility.

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