Testisin, a New Human Serine Proteinase Expressed by Premeiotic Testicular Germ Cells and Lost in Testicular Germ Cell Tumors

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ABSTRACT

We have cloned and characterized a cDNA encoding a new human serine proteinase, testisin, that is abundantly expressed only in the testis and is lost in testicular tumors. The testisin cDNA was identified by homology cloning using degenerate primers directed at conserved sequence motifs within the catalytic regions of serine proteinases. It is 1073 nucleotides long, including 942 nucleotides of open reading frame and a 113-nucleotide 3' untranslated sequence. Northern and dot blot analyses of RNA from a range of normal human tissues revealed a 1.4-kb mRNA species that was present only in testis, which was not detected in eight of eight testicular tumors. Testisin cDNA is predicted to encode a protein of 314 amino acids, which consists of a 19-amino acid (aa) signal peptide, a 22-aa proregion, and a 273-aa catalytic domain, including a unique 17-aa COOH-terminal hydrophobic extension that is predicted to function as a membrane anchor. The deduced amino acid sequence of testisin shows 44% identity to prostatin and contains features that are typical of serine proteinases with trypsin-like substrate specificity. Antipeptide antibodies directed against the testisin polypeptide detected an immunoreactive testisin protein of Mr, 35,000–39,000 in cell lysates from COS-7 cells that were transiently transfected with testisin cDNA. Immunostaining of normal testicular tissue showed that testisin was expressed in the cytoplasm and on the plasma membrane of premeiotic germ cells. No staining was detected in eight of eight germ cell-derived testicular tumors. In addition, the testisin gene was localized by fluorescence in situ hybridization to the short arm of human chromosome 16 (16p13.3), a region that has been implicated in the localization of the testisin gene to chromosome 16 (16p13.3), a region of the genome that is subject to loss of heterozygosity and rearrangement in human testicular cancers, suggesting a potential role for testisin as a tumor suppressor in testicular cancer.

INTRODUCTION

Spermatogenesis, the tightly regulated and dynamic process of male germ cell maturation, occurs in the testis within seminiferous tubules and results in the transformation of a mitotic spermatogonia into a haploid spermatozoan (1). Testicular germ cell tumors arise from immature male germ cells and may differentiate along pathways resulting in several different histological patterns (e.g., seminoma, teratoma, yolk sac tumor, and mixed germ cell tumor). Individual tumors exhibit one or more of these histological patterns. In contrast to many other malignancies, underlying genetic mechanisms in testicular tumorigenesis have not been substantially defined. Familial forms of testicular carcinoma are rare (2), but studies of sporadic testicular tumors have demonstrated loss of heterozygosity on 5q, 11p15.5, 11q13.1, 13q.3, and 16p13.3 (3), suggesting that these regions may contain candidate tumor suppressor genes.

Testicular germ cell maturation is dynamic, requiring cell-cell communication and localized cell-extracellular matrix interactions (4). Regulation of such processes involves cell surface proteolysis, which is important not only for matrix remodeling but also for regulation of growth and differentiation through activation and/or release of functionally diverse effector molecules, including cytokines, growth factors, and cell surface receptors. Not only is characterization of cell surface proteolysis important for understanding germ cell maturation, but cell surface proteinases also constitute potential new targets for anticancer therapies.

The serine proteinases are a large multigene family, the members of which participate in proteolytic reactions that are essential to a diverse range of physiological and pathological processes (5). These enzymes are generally expressed as inactive zymogens; activation results in rapid molecular responses without the requirement for de novo protein synthesis. The involvement of serine proteinases during the later stages of male germ cell maturation and in fertilization has been documented. The testis-specific serine proteinases human acrosin (6) and mouse TESP-1 and TESP-2 (7) are recognized as playing roles during the final stages of sperm development. Additionally, the prostate epithelial cell serine proteinase, PSA, catalyzes the liquefaction of seminal coagulum (8). Plasminogen activators have been implicated in the degradation of tight junctions in the seminiferous tubules of rat testes (9). Furthermore, as yet uncharacterized serine proteinases are also present on sperm cells as they pass through the epididymis and are necessary for the segregation of sperm surface proteins into distinct domains and the attainment of fertilization competence (10, 11).

The enzymatic properties of serine proteinases are dependent on a catalytic triad of His, Asp, and Ser amino acids (12), which are present in motifs that are highly conserved among family members. We have exploited this property in the present study by using a “homology cloning” strategy (13–15) to identify a novel serine proteinase. Testisin is the first serine proteinase to be identified that is expressed by germ cells prior to the first meiotic division and likely functions in proteolytic reactions that are associated with male germ cell maturation. Its loss of expression by testicular tumors of germ cell origin and the localization of the testisin gene to chromosome 16 (16p13.3), a region of the genome that is subject to loss of heterozygosity and rearrangement in human testicular cancers, suggest a potential role for testisin as a tumor suppressor in testicular cancer.

MATERIALS AND METHODS

Homology Cloning of Testisin cDNA. Homology cloning was performed by reverse transcription-PCR using degenerate oligonucleotides directed at

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conserved regions of serine proteinases (13–15). Total RNA (5 μg), isolated from the human cervical adenocarcinoma cell line HeLa S3 (ATCC CCL 2.2) by the method described previously (16), was reverse transcribed at 42°C using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) in the presence of oligo(dT)12-18 (0.25 μg/μl; Pharmacia Biotech, Uppsala, Sweden), 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 10 mM dTT, and 0.5 mM spermidine in a total volume of 20 μl. PCR was performed using 1 μl of the reverse transcriptase reaction mixture, 500 ng of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, and 1–2 units of Taq polymerase (Perkin-Elmer, Norwalk, CT). The primers were: forward, 5'-ACAGATTTCTGTTGTTGCGCAGTGTG3'; and reverse, 5'-ACAGAATTCAGGGGCGGCGA-3' (X = A or G, Y = C or T, lowercase). Cycling conditions were as follows: 94°C for 2.5 min, 35°C for 10 cycles, followed by 22°C for 3 min; followed by 33 cycles of 94°C for 2.5 min, 57°C for 2.5 min, and 72°C for 3 min; and a final extension at 72°C for 7 min. PCR products of ~450 bp were ligated into pGEM-T (Promega), cloned, and analyzed by DNA sequencing. A DNA fragment was identified that represented a partial testis cDNA (nucleotides 267–723). The 3' end of full-length testis cDNA (Clone U, encoding nucleotides 347–1073) was obtained by screening a human HeLa cell Uni-ZAP XR cDNA library (Stratagene, La Jolla, CA) with the radiolabeled partial testis cDNA fragment obtained by reverse transcription-PCR. Clones encoding the 5' end of full-length testis cDNA were obtained both by 5' RACE and screening of the HeLa cell library by PCR. 5' RACE (Life Technologies, Inc., Gaithersburg, MD) was performed using the nested primers 5'-TCTGTCGGTTGTCTCAAA-3' and 5'-CGAAGTTAAGGGTTAGTAG-3' and the supplied anchor primer. The longest clone obtained, R1-2, spanned nucleotides 34–379 of the full-length cDNA. PCR screening of the HeLa cell library was performed using two rounds of amplification with the same nested gene-specific primers and a vector-specific oligonucleotide (T, 5'-ATACCCCTCACA- AAGGGA-3'). Step-down cycling conditions were: 95°C for 10 min; 3 cycles at each annealing temperature of 95°C for 30 s, 70 – 62°C in 2°C steps for 30 s, and 72°C for 3 min; followed by 18 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 3 min; and a final extension at 72°C for 7 min. The longest clone obtained, 5-4, encoded nucleotides 1–527 of the full-length cDNA.

**Plasmid Construction.** A plasmid containing full-length testisin [testisin(L)] cDNA was generated by ligating a BglII-XbaI DNA fragment from clone54intoBglII-XbaI-digestedclone U, generating pBluescriptHEL A2(L)B65. A Kast-Msci DNA fragment from clone R1-2 was ligated into the Kast-Msci-digested pBluescriptHEL A2(L)B65 to generate pBluescriptHEL A2(S)B41, the construct encoding the short isoform, testisin(S).

A SaclI fragment of pBluescriptHEL A2(S)B41 encoding amino acids 90–279 was cloned into the BamHI site of pGEX-1 (Pharmacia Biotech) to generate a partial GST-testisin fusion construct for expression in *Escherichia coli.*

For expression in eukaryotic cells, a DNA fragment encoding nucleotides 13–954 of the testisin(S) isoform from pBluescriptHEL A2(S)B41 was generated by PCR using the following primers: forward, 5'-GCAGGCTGAGAGAGGGCCGCAGC-3'; and reverse, 5'-GCAACTCTAGATCGTCGTTGGTGGTGTTGGTGCAGGAGTGG-3'. The amplification product was cloned into pGEM-T (Promega). The fragment was then excised with NotI and cloned into the NotI site of pcDNA3 (Invitrogen, Carlsbad, CA), generating pcDNA3Test(S-C). An expression vector containing the long isoform, pcDNA3Test(l-C) encoding nucleotides 13–960 of the testisin(L) was generated using the same procedure from pBluescriptHEL A2(L)B65.

All constructs were verified by DNA sequence analysis.

**Patients and Tumor Specimens.** Eight paired samples of testicular tumors and adjacent unaffected testicular tissue were obtained at the time of surgery and were snap-frozen in liquid nitrogen for RNA analyses. In addition, portions of unaffected testis, tumor tissues, and junctional specimens (at the tumor and unaffected testis tissue interface) were processed for routine histological examination by paraffin-embedding formalin-fixed tissue. Histological evaluation was based on routine pathology reports and included four seminomas, one teratoma, one yolk sac tumor, and two mixed germ cell tumors. Normal testicular tissue was also obtained from a patient undergoing bilateral orchiectomy for treatment of prostatic carcinoma.

**Northern and Poly(A)⁺ RNA Dot Blot Analyses.** Total RNA isolated from frozen tissue specimens and the cell lines HeLa S3 and SW480 (ATCC CCL 228), was separated by denaturing gel electrophoresis and transferred to Hybond-N nylon membranes (Amersham, Aylesbury, United Kingdom) as described (16). Human multiple-tissue Northern blots and a human multiple-tissue dot blot (Clontec, Palo Alto, CA) were obtained commercially. The multiple-tissue Northern blot contained 2 μg of poly(A)⁺ RNA per lane. The dot blot contained poly(A)⁺ RNA from 50 normal adult and fetal tissues normalized to the mRNA expression levels of eight different housekeeping genes and ranged from 89 to 514 ng. Blots were hybridized with [³²P]CTP-labeled msgl-BanⅠ testisin(L) fragment (nucleotides 321–861) in ExpressHyb (Clontec) solution at 65°C and washed to a final stringency of 0.2× SSC-0.1% SDS at 65°C. The dot blot was washed to a final stringency of 0.1× SSC-0.5% SDS at 60°C. Blots were reprobed with β-actin cDNA or an oligonucleotide probe for 18S rRNA (16) to confirm RNA loading in each lane.

**Production of Affinity-purified Antipeptide Polyclonal Antibodies.** Rabbit polyclonal antibodies were generated against testisin-specific peptides derived from nonhomologous hydrophilic regions within the catalytic domain of testisin. Two peptides, each containing a cysteine residue incorporated at the COOH terminus, were synthesized (Auszep, Parkville, Australia) and conjugated to keyhole limpet hemocyanin using μ-maleimidobenzoic acid N-hydroxysuccinimide ester. The peptide sequences were as follows: T175-190, Gly-Tyr-Ile-Lys-Gly-Asp-Glu-Ala-Leu-Pro-His-Thr-Leu-Gln-Cys; and T46-63, Glu-Asp-Ala-Glu-Leu-Gly-Arg-Trp-Pro-Trp-Gln-Gly-Ser-Leu-Arg-Leu-Glu-Trp-Asp-Cys (short isoform numbering). Rabbit antiserum was peptide affinity-purified using SulfoLink coupling gel (Pierce, Rockville, IL). The specificity of each antibody was tested against the immunogenic peptide by ELISA and against recombinant testisin by Western blot.

**Western Blot Analysis.** Proteins were separated by SDS-PAGE on 10–12% gels and transferred electrophoretically to Hybond-P membranes (Amersham). Membranes were blocked with 5% nonfat skim milk powder in Tris-buffered saline (10 mM Tris-HCl (pH 7.0)-150 mM NaCl), incubated with affinity-purified antipeptide antibody and then with horseradish peroxidase-conjugated sheep antirabbit immunoglobulin secondary antibody, and visualized by enhanced chemiluminescence (Amersham).

**Immunohistochemistry.** Paraffin sections (5 μm) of Bouin’s-fixed normal human testis tissue or formalin-fixed tissues from testicular cancer patients were deparaffinized and then rehydrated before antigen retrieval in boiling 10 mM citric acid buffer (pH 6). After cooling, endogenous peroxidase activity was inhibited by a 10-min incubation in 1% hydrogen peroxide. Non specific antibody binding was blocked by incubating the sections in 4% nonfat skim milk powder in Tris-buffered saline (10 mM Tris-HCl (pH 7.0)-150 mM NaCl), incubated with affinity-purified antipeptide antibody and then with horseradish peroxidase-conjugated sheep antirabbit immunoglobulin secondary antibody, and visualized by enhanced chemiluminescence (Amersham).

**RESULTS**

**Isolation of Human Testisin cDNA by Homology Cloning.** PCR amplification of cDNA was performed with degenerate primers designed to anneal to cDNA encoding the conserved regions surrounding the catalytic histidine and serine amino acids of serine proteinases. The deduced amino acid sequence of one amplified product showed high homology to the family of serine proteinases. Extended cDNA sequence of this clone was obtained by library screening and 5’
RACE. The final nucleotide sequence revealed that testisin cDNA was 1073 nucleotides long and included an 18-nucleotide 5′ untranslated region, an open reading frame of 942 nucleotides, and a 113-nucleotide 3′ untranslated region with a polyadenylation signal 34 nucleotides upstream from the poly(A) sequence (Fig. 1). The nucleotide sequence surrounding the proposed start codon showed good agreement with the Kozak consensus for eukaryotic translation initiation (18). The deduced amino acid sequence of the open reading frame predicted a polypeptide of 314 amino acids, including pre-, pro-, and catalytic regions, with a Mᵦ of 34,800 and possessing three potential N-glycosylation sites at Asn²⁴, Asn²⁰⁰, and Asn²⁷³ (Fig. 1). In vitro transcription/translation of testisin cDNA generated a protein product of Mᵦ ~35,000 (data not shown), demonstrating that the predicted start codon was functional in vitro. A hydrophathy plot (19) of the amino acid sequence revealed a hydrophobic region located at amino acid positions 1–19 that conformed with the consensus for a typical NH₂-terminal secretory signal peptide (20). Thus, this region is likely to function as a signal peptide, directing the newly synthesized protein to enter the endoplasmic reticulum. Unusually for members of the serine proteinase family, a second hydrophobic region was identified at the COOH terminus of testisin at amino acids 298–314. This COOH-terminal extension likely constitutes a membrane anchor, as has been reported for the serine proteinases prostasin (21) and CAP1 (22).

Comparison of the predicted testisin polypeptide with protein databases showed that it possessed the hallmark features of the serine proteinase family. It is a putative zymogen containing pro- and catalytic regions of 22 and 273 residues, respectively (Fig. 1), delineated by a classic serine proteinase activation motifArg⁴¹-Ile-Val-Gly-Gly, with cleavage likely occurring between Arg⁴¹ and Ile⁴₂. The catalytic region includes the triad of His⁸², Asp₁₃⁷, and Ser₂₃₈ residues in positions and surrounding motifs that are required for catalytic activity of serine proteinases. Ten Cys residues occur in conserved positions. By analogy to other serine proteinases, eight of these are likely to function to form disulfide bridges within the catalytic region, and the remaining two are likely to link the pro- and catalytic regions (Fig. 1).

cDNAs encoding two testisin isoforms, designated testisin(S) and testisin(L), were identified. These two isoforms differed by six nucleotides, CTATAG, at nucleotide position 276 (Fig. 1). This six-nucleotide insertion incorporates a consensus 3′ splice site, and the position of this insertion within the cDNA sequence is consistent with the position of an intron-exon junction in other serine proteinase genes of the chymotrypsin family (23–25), suggesting that these isoforms may have arisen as a result of alternative mRNA splicing. The presence of these six nucleotides results in the insertion of two amino acids Tyr⁹⁷-Ser in the polypeptide sequence, only four amino acids distant from the catalytic His⁸². The functional significance of this insertion to the catalytic activity and/or substrate specificity of testisin is not yet known.

Testisin mRNA Is Strongly Expressed Only in Testis. Northern blot analysis showed that testisin mRNA of ~1.4 kb was expressed constitutively in HeLa cells, whereas no expression was detected in the colon cancer cell line SW480 (Fig. 2A). The distribution of testisin mRNA in a range of normal human tissues was examined by Northern blot analysis. Abundant expression of testisin mRNA was detected only in the testis (Fig. 2C), with prolonged exposures showing barely detectable signals in salivary gland, bone marrow, lung, and trachea.

Homology of Testisin with Other Serine Proteinases. The predicted amino acid sequence of testisin shares 44% identity to human prostasin (21), 39% to Xenopus CAP1 (22), 38% to human hepsin (26), 36% to human acrosin (27), 30% to human chymotrypsin (28), and 26% to PSA (8). Alignment of testisin with the sequences of several serine proteinases reveals a number of common features (Fig. 3). The catalytic triad of His⁶², Asp¹₃⁷, and Ser²₃₈ and their surrounding motifs are in positions that are highly conserved among other serine proteinases. The presence of Asp²³⁵ at the bottom of the serine proteinase binding pocket six residues before the active site (Ser²³⁸) predicts that testisin has trypsin-like specificity with proteolytic cleavage after Arg or Lys residues in target substrates. A conserved Ser²²⁷-Trp-Gly motif is predicted to be located at the top of the binding pocket in testisin and is likely to be involved in correct orientation of the scissile bond of the substrate.

Expression of Recombinant Testisin in Bacteria and Eukaryotic Cells. Recombinant testisin was expressed in E. coli as a GST-testisin fusion protein. Fig. 4A shows a Western blot of the GST-testisin fusion protein following induction and purification from E. coli. The fusion protein was immunoreactive against affinity-purified-antibodies generated against the testisin-specific peptide (T175-190).

Transient expression of testisin from cDNA under the control of the constitutive cytomegalovirus promoter was demonstrated in COS-7 cells. Maximal testisin protein expression was observed 36 h after

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Footnote:

¹ The nucleotide sequence reported in this paper has been deposited in the DDBJ/GenBank/EMBL database (accession no. AF058300; deposited April 8, 1998).
progressively increased with stage, with the most intense immune-specific staining seen in late pachytene and diplotene spermatocytes (Fig. 5A). Staining was diffuse within the cytoplasm of these cells with a corresponding accentuation of the plasma membrane, consistent with the identified COOH-terminal extension being involved in anchoring of testisin on the cell surface. In addition there was intense, focal, cell surface staining at some spermatocyte junctions (Fig. 5C). Some spermatocytes also showed evidence of dense, crescent-shaped transient transfection, with an immunoreactive band detected at Mr 35,000–39,000 (Fig. 4B). Because the mature testisin zymogen would be predicted to migrate at Mr 32,700, these data indicate that testisin undergoes a posttranslational processing event, such as N-glycosylation. Testisin was undetectable in conditioned media from these cells, demonstrating that testisin was predominantly cell-associated (data not shown).

**Fig. 2. Testisin mRNA expression.** A, Northern analysis of total RNA isolated from HeLa and SW480 cells, probed with 32P-labeled testisin or labeled 18S rRNA as a loading control. B, Northern blot analysis of mRNA blots probed with 32P-labeled testisin cDNA or labeled β-actin cDNA. C, graphical representation of signal intensities obtained following hybridization with 32P-labeled testisin cDNA of a commercial poly(A)+ RNA dot blot containing 50 normal human tissues. The units on the Y axis are arbitrary.

**Testisin Is Associated with Primary Spermatocytes during the First Meiotic Prophase.** Maturation of male germ cells proceeds through several ordered stages, with maturation occurring from the base of the seminiferous tubules toward the tubule lumen. Committed spermatogonia undergo two rounds of meiotic division, passing in the first meiotic prophase, sequentially through preleptotene, leptotene, zygotene, pachytene, and diplotene stages, during which chromosome pairing and cross-over events occur. Following the first meiotic division, the resultant secondary spermatocytes proceed through a second meiotic division to become haploid round spermatids, which are further processed through a continuum of gross morphological changes to elongated sperm (1). To begin to understand the function of testisin in human testis, we examined testisin protein expression by immunohistochemical analysis of normal human adult testis. Testisin expression was first seen in zygotene spermatocytes and staining progressively increased with stage, with the most intense immune-specific staining seen in late pachytene and diplotene spermatocytes (Fig. 5A). Staining was diffuse within the cytoplasm of these cells with a corresponding accentuation of the plasma membrane, consistent with the identified COOH-terminal extension being involved in anchoring of testisin on the cell surface. In addition there was intense, focal, cell surface staining at some spermatocyte junctions (Fig. 5C). Some spermatocytes also showed evidence of dense, crescent-shaped transient transfection, with an immunoreactive band detected at Mr 35,000–39,000 (Fig. 4B). Because the mature testisin zymogen would be predicted to migrate at Mr 32,700, these data indicate that testisin undergoes a posttranslational processing event, such as N-glycosylation. Testisin was undetectable in conditioned media from these cells, demonstrating that testisin was predominantly cell-associated (data not shown).

**Fig. 3. Alignment of testisin with closely related serine proteinases.** Residues which are identical between at least three of the serine proteinases are boxed; disulfide bond-forming cysteines; catalytic residues. Dashes represent gaps for alignment purposes. The COOH-terminal extension of acrosin has been truncated in this figure. GenBank database accession nos. are as follows: prostasin, U33446; acrosin, Y00970; hepsin, X07732; PSA, X05332.

**Fig. 4. Expression of recombinant testisin.** A, Western blot of expression in *E. coli* of the recombinant GST-testisin90–279 fusion protein. Lane 1, untransformed *E. coli* DH5α cells; Lane 2, *E. coli* DH5α cells transformed with GST-testisin90–279 expression construct (uninduced); Lane 3, *E. coli* DH5α cells transformed with GST-testisin90–279 expression construct (induced with 0.5 mM isopropyl-β-thiogalactopyranoside for 3 h); Lane 4, GST-testisin90–279 fusion protein affinity-purified on Glutathione Sepharose 4B (Pharmacia Biotech). B, Western blot of transiently transfected testisin in COS-7 cells. Approximately 5 × 10⁶ COS-7 cells were transfected with pcDNA3Test(S-C) by electroporation and harvested after the times indicated. Mock-transfected cells were transfected with vector alone for 24 h. Both blots were probed with antitestisin(T175-190) polyclonal antibody.
compartmentalized staining as shown in Fig. 5D. No detectable staining was seen in spermatogonia, spermatids, Sertoli cells, or other cells of the testicular interstitium. Control experiments using the T175-190 polyclonal antibody in the presence of competing T175-190 peptide showed absence of this specific staining pattern (Fig. 5B). An identical, albeit weaker, staining pattern was observed in experiments performed using a testisin-specific antibody generated against a testisin peptide, T46-63 (data not shown).

**Testisin Expression Is Lost in Testicular Tumors.** Because the vast majority of testicular cancers arise from male germ cells and because premeiotic germ cells have been hypothesized to be precursors for male germ cell tumors (29, 30), testisin expression was examined in a series of these cancers. Northern blot analysis of RNA isolated from eight paired specimens of testicular tumor and unaffected testis showed strong signals for testisin mRNA in all unaffected samples; no signal was detected in any corresponding testicular tumors (Fig. 6). Immunohistochemical staining for testisin protein expression in each of these patients confirmed a corresponding loss of protein expression in each tumor, whereas normal testisin expression was detected in the adjacent unaffected testis tissue. Representative examples are shown in Fig. 5, E–I. Fig. 5E shows testisin staining in an unaffected seminiferous tubule of patient 795, showing predominant testisin staining in pachytene spermatocytes, similar to that observed in normal testis. No testisin staining was detected in the mixed germ cell tumor from this patient (Fig. 5F). Fig. 5G shows testisin staining, which is typical for normal testis, in an unaffected tubule of patient 798, with a corresponding loss of testisin expression in the seminoma from this patient (Fig. 5H). It was generally observed that testisin expression was normal in morphologically normal tissue adjacent to the tumor, as illustrated in the tumor/unaffected tissue junction from patient 798 (Fig. 5I). These data suggest that loss of testisin expression is associated with testicular germ cell tumor formation and/or progression.

**Localization of the Testisin Gene by FISH.** The chromosomal localization of the human testisin gene was mapped by FISH to human metaphase chromosomes from two normal males. Analysis of 20 metaphase chromosomes from one normal male showed strong signal on one or both chromatids of chromosome 16 in the region 16p13.2–16pter; 87% of this signal was at 16p13.3 (Fig. 7). There was a total of 8 nonspecific background dots observed in these 20 metaphases. A similar result was obtained from hybridization of the probe to 15 metaphases from the second normal male (data not shown).
Fig. 6. Testisin mRNA is lost in testicular tumors. Northern blot analysis of paired unaffected testis (Lanes N) and primary testicular tumors (Lanes T) analyzed using radiolabeled full-length probe encoding testisin cDNA. Testicular cancer specimens 438, 616, 788, and 798 were seminomas; 783 was a mixed germ cell tumor; 1007 was a yolk sac tumor; and 619 and 795 were teratomas. Lane P: a section of normal testis obtained following bilateral orchidectomy for treatment of prostate cancer. As a control for the amount of RNA loaded in each lane, each blot was reprobed using a radiolabeled oligonucleotide directed against 18S rRNA.

DISCUSSION

Testicular tumors are rare, comprising 2% of all cancers in men; however, testicular cancer is the most common malignancy affecting males aged 20–35 years (31). Because the molecular basis of these cancers is not known, identification of cellular genes involved in testicular tumorigenesis will increase our understanding of development of testicular tumors and may provide the basis for new targeted therapies. In this study, we have characterized a novel cDNA, testisin, which encodes a serine proteinase that is strongly and specifically expressed by maturing primary spermatocytes prior to the first meiotic division. In addition to the loss of expression of testisin in germ cell tumors, localization of the testisin gene at chromosome band 16p13.3 suggests a potential role in suppression of tumorigenesis of testicular germ cell tumors.

Although the sequence of testisin is unique, homology comparisons showed testisin was a member of the chymotrypsin (S1) family of serine proteinases. These serine proteinases include a signature catalytic triad of His, Asp, and Ser amino acids and are generally produced as inactive zymogens that are activated following proteolytic cleavage. Testisin contains a characteristic serine proteinase activation motif (RIVGG) with cleavage predicted to occur following Arg41. This would release a preregion linked by a disulfide bond to the catalytic domain, which is typical of many of the members of this family. On the basis of the presence of an acidic amino acid, Asp, in the putative substrate binding pocket, active testisin would be predicted to cleave its target substrate with specificity for the basic amino acids, Arg or Lys (12).

Two isoforms of testisin, differing by two amino acids located near the catalytic histidine, result from a six-nucleotide insertion. The existence of these isoforms may be evidence of intron-exon junctional sliding (32). The additional two amino acids occur in a loop that, in other serine proteinases, stabilizes primary interactions between surrounding structures of the proteinase and the substrate (12). Although this insertion is unlikely to influence the primary specificity of testisin for basic amino acids, it is possible that secondary effects, such as substrate affinity, may be different between the two isoforms.

Testisin includes hydrophobic regions at the NH2 and COOH termini that are predicted to act as secretory and membrane attachment signals, respectively. Testisin is only the second human serine proteinase described with a hydrophobic COOH-terminal extension. The catalytic region of the vast majority of serine proteinases forms the COOH terminus, and most serine proteinases are either secreted or targeted to cytoplasmic storage organelles by an NH2-terminal signal sequence. Recently, however, some membrane-anchored serine proteinases have been identified in different species, including, in addition to testisin, human prostasin (20), mouse TESP-1, and mouse TESP-2 (6), each of which possess COOH-terminal amino acid extensions. The COOH-terminal extension of prostasin is believed to anchor prostasin to the plasma membrane of prostate epithelial cells, from which it may be proteolytically released into the semen (21). TESP-1 and TESP-2 are thought to be anchored to the cell membrane via a glycosyl-phosphatidylinositol linkage (7). Like these proteinases, testisin appears to be present on the plasma membrane, possibly attached via a glycosyl-phosphatidylinositol anchor.

The restricted expression pattern of testisin is consistent with a specialized role during male germ cell development. In the testes, testisin is expressed exclusively by primary spermatocytes, with intense staining in germ cells prior to the first meiotic division. This is the first described serine proteinase strongly expressed at such an early stage in germ cell development. Other described germ cell serine proteinases, acrosin (27), TESP-1, and TESP-2 (7), are synthesized at a much later stage and are present in mature sperm. Thus, testisin may represent a component of an as yet unrecognized proteolytic cascade involved in germ cell maturation, analogous to the fibrinolytic and coagulation cascades.

The physiological function of testisin is not yet known. Proteolysis is important for proliferation, apoptosis, differentiation, and cell migration; all processes that are integral to normal germ cell development. In testis, diploid spermatogonia differentiate into haploid spermatozoa following successive rounds of mitotic and meiotic cell divisions and extensive morphological restructuring. Biochemical events occurring during meiosis are poorly defined, although it is known that processes such as chromatin condensation, formation of synaptonemal complexes, and genetic recombination are proceeding (33). We hypothesize that loss of testisin expression may alter differentiation of immature germ cells and/or lead to arrest of testicular germ cell maturation and unregulated proliferation. Testisin could participate in proteolytic events required for migration of maturing germ cells in the adluminal space of the seminiferous tubule or in matrix remodeling. Alternatively, because exchange of soluble factors...
and coordinated cell surface interactions between developing germ cells and Sertoli cells are essential for spermatogenesis (34), testisin may participate in proteolytic cleavage and release of specific factors and/or activation of bioactive molecules. Such events may be essential for normal meiotic cell division in spermatogenesis and, clearly, have implications for abnormalities in germ cell maturation, such as those that occur in sterility, fertility, and testicular cancer.

The testisin gene has been localized near the telomere of human chromosome 16, at 16p13.3. This region of human chromosome 16 is associated with high genetic instability; documented rearrangements underlie a variety of common human genetic disorders, including α-thalassemia, polycystic kidney disease, tuberous sclerosis, familial Mediterranean fever, and Rubenstein-Taybi syndrome (35). Loss of heterozygosity studies have identified 16p13.3 as a potential locus for a tumor suppressor gene associated with male germ cell tumors (2). It remains to be determined whether testisin functions as a tumor suppressor gene. However, the demonstrated loss of testisin mRNA and protein expression in testicular cell lines (36). Expression of NES1 has recently been shown to inhibit mammary epithelial cells and is down-regulated in most breast cancer parallels that proposed for the recently identified serine proteinase, nor-

ular germ cell tumors implies its absence may contribute directly or indirectly to testicular tumor development or progression. Such a role parallels that proposed for the recently identified serine proteinase, normal epithelial cell specific-1 (NES1). NES1 is expressed in normal mammary epithelial cells and is down-regulated in most breast cancer cell lines (36). Expression of NES1 has recently been shown to inhibit anchorage-independent growth and suppress oncogenicity in nude mice (37), indicating a tumor suppressor role for this serine proteinase.

Taken together, our data support a role for testisin in testicular germ cell maturation and, possibly, in the initiation and/or progression of testicular cancers. The restricted expression of testisin and the similarities between testisin and other known serine proteinases suggest that testisin may have a unique and critical biological function in germ cell growth and/or differentiation in the testis.

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Testisin, a New Human Serine Proteinase Expressed by Premeiotic Testicular Germ Cells and Lost in Testicular Germ Cell Tumors


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