A Novel N-Terminal Domain Directs Membrane Localization of Mouse Testis-Specific Calpastatin

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ABSTRACT

Multiple isoforms of calpastatin have been identified with unique N-terminal regions followed by identical calpain inhibitory domains (II–IV). In many instances the isoforms are cell-type specific, although the precise functional differences among these N-terminal regions are largely unknown. Here we report a germ cell-specific isoform of calpastatin (tCAST) that consists of a novel N-terminal peptide of 40 amino acids (domain T) followed by domains II to IV of somatic calpastatin (sCAST). Domain T is responsible for membrane association of tCAST through a protein modification by myristylation. Mutation of the myristylation site eliminates membrane targeting. Unlike most of the isoforms of calpastatin that are generated through alternative RNA splicing or post-translational proteolysis, the testis-specific isoform is transcribed from an intronic promoter in haploid germ cells of the tests. The intronic promoter directs specific expression of a reporter transgene in developing germ cells of the mouse testis.

gene regulation, spermatid, spermatogenesis, testes

INTRODUCTION

Calpastatin is an endogenous inhibitor of calpain, a ubiquitous calcium-dependent cysteine protease. The calpain-calpastatin system has been detected in the cytoplasm in numerous mammalian cell types and found to play important roles in many physiological processes, including activation of various enzymes [1], regulation of cell cycle progression [2], apoptosis [3], and membrane fusion [4]. Analysis of cDNA sequences of calpastatin from several species reveals a five-domain structure in which a leading domain L is followed by four repetitive domains (I–IV), each of which has inhibitory activity against calpain in the presence of calcium [5–8]. A number of isoforms that are generated by alternative splicing or post-translational proteolysis have been identified in various species [9–11]. These isoforms exhibit cell-type-specific usage of certain exons in domain L and I. Recently, Cong et al. [12] and Takano et al. [13] reported the presence of a new isoform of calpastatin derived from the usage of an upstream promoter and translation start codon. Although these tissue-specific N-terminal domains possibly are involved in the modulation of calpastatin activity, the physiological significance and the mechanism of regulatory functions remain unknown.

The germinal epithelium of the mammalian testis contains a variety of cell-type- and stage-specific proteins that regulate spermatogenesis and subsequent sperm maturation. Many of these proteins are different from their somatic counterparts in structure and function that result from various mechanisms including activation of a testis-specific member of a gene family [14], alternative splicing [15], or usage of alternative transcription initiation sites [16, 17]. A testis-specific isoform of calpastatin (tCAST) has been previously identified from a human testis cDNA library with a unique N-terminal peptide of 40 amino acids followed by C-terminal domains II–IV [18]. The physiological significance of the N-terminal domain in regulating calpastatin activity is not clear. Analysis of genomic sequences surrounding the testis exon reveals that it is localized within intron 14 of the somatic calpastatin (sCAST) gene, reminiscent of the gene structure of testis-specific angiotensin-converting enzyme (tACE) [16, 19], calsermin [17], and c-kit [20], each of which is generated from a promoter within an intron of its somatic gene.

In this report, we show that mouse tCAST is transcribed from an intronic promoter in haploid germ cells and that the promoter is able to direct germ cell-specific expression of a reporter gene in transgenic mice. Unlike somatic calpastatin that remains primarily in the cytosol, tCAST is found in both cytosol and membrane fractions. Further analysis reveals that a modification by myristic acid within domain T is responsible for the membrane association of tCAST.

MATERIALS AND METHODS

Constructs

The promoter region of mouse tCAST from –940 to +52 (the major transcription start site is designated as +1) was amplified by polymerase chain reaction (PCR) and subcloned into a LacZ reporter vector pNAss β to generate pNAss β mtCT for transgenic study. The full-length mouse tCAST cDNA was subcloned into a eukaryotic expression vector (pcDNA3, abbreviated as C3) to produce C3tCAST for expression in mammalian cells. A single glycine (+2) to alanine mutation was introduced into C3tCAST by PCR to obtain C3tCAST G2A. The GFP (green fluorescence protein) fusion proteins, TE-GFP and TE-GFP G2A, were constructed by cloning PCR fragments corresponding to Met (+1) to His (+40) of mouse tCAST protein into a GFP reporter vector (pEGFP-N2, Clontech, Palo Alto, CA). The His-tag recombinant protein of mouse calpastatin domain IV was generated from a bacterial expression vector (pQE30, Qiagen, Valencia, CA) containing mouse tCAST amino acids 276 to 430. The purified protein was used to immunize rabbits to obtain polyclonal antiserum against mouse calpastatin domain IV.

Cell Culture and Transient Transfection

HeLa cells were maintained in Dulbecco modified Eagle medium with 10% fetal calf serum. Aliquots of 2 × 10^5
cells were seeded on a coverslip and transfected with SuperFect (Qiagen). Twenty hours later, cells were fixed in 3.7% formaldehyde. After three washes in PBS, cells were mounted in phenylenediamine dihydrochloride solution and visualized with deconvoluting microscopy.

**Cellular Fractionation**

Transfected cells were homogenized in a homogenization buffer containing 50 mM Tris, pH 7.4, 1 mM EGTA, 2.5 mM EDTA, 1 mM dithiothreitol (DTT), 150 mM NaCl, 0.5 mM PMSF, 0.5 mM benzamidine, 1 μg/ml leupeptin, and 0.2 U/ml aprotinin. Cell lysates were centrifuged at 100,000 x g to remove nuclei and intact cells. The postnuclear supernatant was further separated into the supernatant (S100, the cytosol fraction) and the pellet (P100, the membrane fraction) by centrifugation at 100,000 x g for 1 h. An equal volume of the homogenization buffer containing 1% Triton X-100 was added to solubilize the pellet at 4°C. Equal volumes of S100 and P100 were loaded onto SDS-PAGE gels and analyzed by Western blot.

**Labeling with [3H]Myristic Acid**

Cells were metabolically labeled as described previously [21] and then lysed in RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EGTA, 2.5 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.5 mM PMSF, 0.5 mM benzamidine, 1 μg/ml leupeptin, and 0.2 U/ml aprotinin). The tCAST was precipitated by incubation with anti-mouse calpastatin domain IV antiserum and subsequently with Protein A Sepharose beads (Sigma, St. Louis, MO). Immunoprecipitated proteins were resolved by SDS-PAGE and detected by fluorography following the treatment of the gel with EN3 HANCE (NEN, Boston, MA).

**RNA Preparation and Analysis**

Total RNA was isolated from tissues using TriReagent (Sigma). Poly(A) RNA was further purified using the PolyATtract mRNA Isolation System (Promega, Madison, WI).

For primer extension assay, a 25-base pair (bp) oligonucleotide complementary to the sequences −2 to +23 with respect to ATG was end labeled with [γ-32P]ATP and used to prime poly(A) RNA. Single-stranded cDNA was synthesized with reverse transcriptase and resolved on a sequencing gel.

The S1 nuclease analysis was performed using the same end-labeled oligonucleotide that was extended on a plasmid DNA containing sequences up to −300 bp 5' to the ATG. The double-stranded DNA thus generated was then digested with KpnI and separated on a 1.2% alkaline agarose gel. A single-stranded antisense DNA probe was recovered from the gel and hybridized to the poly(A) RNA. The hybrid was incubated with 100 units of S1 nuclease, and protected DNA fragments were analyzed on a sequencing gel.

**In Situ Analysis**

Mouse testes were fixed in Bouin’s and paraffin embedded. In situ hybridization analysis was performed on 5-μm sections as described previously [22]. Sections were rehydrated and hybridized with digoxigenin-labeled riboprobes at 47°C. Slides were then washed to remove nonspecific binding, incubated with alkaline phosphatase-conjugated antibody against digoxigenin, and developed by chromogenic reaction.

**Generation of Transgenic Mice**

Transgenic mice were produced by injecting linearized pNAsβ miCT into pronuclei of one-cell stage embryos. Integration of the transgene was determined by PCR on tail DNA. Whole mount lacZ staining and β-galactosidase activity were performed as described before using the Galacto-Light Plus chemiluminescent reporter assay system from Tropix, Inc. (Bedford, MA) according to manufacturer’s instructions [23]. Briefly, 20 μg of tissue extracts was incubated with reaction buffer for 1 h after heating at 48°C to inactivate endogenous β-gal activity. The chemiluminescent substrate was cleaved by β-gal. The intensity of the reaction is read by a luminometer. Following whole-mount X-gal staining, testes were postfixed in formalin and sections were counterstained with nuclear fast red.

**RESULTS**

**Testis CAST Is Specifically Expressed in Haploid Round Spermatids in Mouse Testis**

Mouse tCAST cDNA was isolated initially by screening a testis cDNA library with a fragment of rat sCAST DNA. A full-length cDNA clone (GenBank aa061542) was obtained by searching a mouse expressed sequence tag (EST) database. Sequence analysis revealed a protein structure consisting of a novel N-terminal peptide of 40 amino acids followed by domains II, III, and IV of sCAST (GenBank AF190151).

Expression of tCAST was detected only in testis (data not shown). To study the expression of tCAST during testis development, RNA isolated from mouse testes at different postnatal stages was hybridized with tCAST cDNA probes. While the somatic isoform is present as early as postnatal Day 10, tCAST cannot be detected until Day 20, coincident with the appearance of round spermatids (Fig. 1A). As expected, in situ hybridization revealed abundant tCAST mRNA in round spermatids but not in earlier stages of spermatogenesis (Fig. 1, B and C). The level of tCAST mRNA increases dramatically in adult testis, corresponding to the increasing percentage of round spermatids.

**Testis CAST Is Transcribed from an Intronic Promoter**

The testis-specific exon is located within intron 14 of the somatic gene in human DNA [18]. Two possible mechanisms by which tCAST could be generated are alternative splicing from the somatic transcript or de novo transcription from an intronic promoter. To distinguish between these two possibilities, primer extension and S1 nuclease analysis were performed to determine the transcription initiation site of mouse tCAST. Primer extension using an oligonucleotide (−2 to +23) (A of ATG is designated as +1) (Fig. 2A) complementary to the 5' end of mouse tCAST mRNA produced a major extended fragment of 77 nucleotides (nt) in testis poly(A) RNA, placing the major transcription start site at 54 bp 5' to the ATG (Fig. 2B). A number of minor fragments ranging in size from 60 to 107 nt were also observed. To test whether tCAST mRNA is generated from preexisting initiation sites, S1 nuclease analysis was performed using an antisense DNA probe corresponding to (−300 to +23). This probe was hybridized to poly(A) RNA purified from testis or liver followed by nuclease digestion (Fig. 2C). The S1 nuclease-resistant fragments displayed a similar pattern with expected sizes, indicating that tCAST transcription is initiated from a novel promoter with the major start site located 54 bp upstream from the ATG.
is unlikely to be an alternatively spliced isoform of the sCAST transcript.

Consistent with the suggestion that tCAST transcription is initiated from intron 14 of the calpastatin gene, the promoter activity of this region was tested in transgenic mice. Four transgenic lines bearing the reporter construct were generated. Activity of β-gal was detected in the testes of the F1 generation from two independent lines (Fig. 3A). In both cases, patchy staining of lacZ activity was observed in the seminiferous tubules. Cross sections of the testes revealed X-gal staining in those cells with smaller nuclei near the lumen of the tubule but not in the cells close to the margin (Fig. 3B), clearly localizing the transgene expression to round spermatids. Assays of β-gal activity confirmed that expression is testis-specific (Fig. 3C). While high-level expression was observed in testis extracts, β-gal activity in other tissues approximated the low levels found in nontransgenic littermates.

Testis CAST Is Different from sCAST by Its Membrane Association

In order to identify functional motifs within domain T, we performed a database search on structural fingerprints and found a putative myristylation site. Because this motif is highly conserved between the human and mouse testis calpastatin isoforms [18], it may be of functional significance. Because most myristylated proteins can be targeted to membranes by myristate [24], we first asked whether tCAST is associated with membranes. Tissue extracts were fractionated into cytosol and membrane fractions and probed by Western blots with antibody raised against mouse calpastatin domain IV. Because calpastatin has been known for its aberrant migration on SDS-PAGE gels [25], protein extracts from HeLa cells transiently transfected with pcDNA3 vector (C3) or pcDNA3 tCAST (C3tCAST) were used to determine the specificity of the antibody and also the size of tCAST on SDS-PAGE gels. A band of 78 kDa was observed only in extracts from cells transfected with the full-length tCAST but not with the vector (Fig. 4, lanes 1–2), thus confirming antiserum specificity for calpastatin. Two immunoreactive proteins were detected in the testis extracts, with the slower migrating band subsequently identified as another isoform of tCAST (data not shown) generated by differential usage of 5' splicing donor sites of the testis exon. While both bands were present in the membrane fraction as well as the cytosol fraction, sCAST was found predominantly in the cytosolic fraction (Fig. 4, lanes 3–6). The differential localization is likely to result from their distinct N-termini.
FIG. 3. Transgenic study of tCAST promoter. A) Whole mount X-gal staining of mouse testes. Compared to the nontransgenic testis, the transgene showed blue staining in some tubules. B) Distribution of the transgene expression in testis. Sections (14 μm) were prepared from stained testis (in A) and counterstained with nuclear fast red. Note the localization of X-gal staining toward to the lumen in the cells with smaller nuclei. C) Testis-specific transgene expression from the intronic promoter. Extracts were prepared from various tissues. Equal amounts of protein were used in the enzyme assay. Open boxes represent control mice, and solid boxes represent transgenic males.

To test the possibility that domain T is responsible for the membrane association of tCAST, we fused domain T to the N-terminus of GFP to generate TE-GFP and examined the subcellular localization of the fusion protein in transiently transfected cells. Compared with GFP, which is largely distributed uniformly in HeLa cells (Fig. 5A), TE-GFP appeared to be primarily cytoplasmic with both punctate and some diffuse staining. Extensive TE-GFP accumulation in the perinuclear region was observed (Fig. 5B). The subcellular localization of this GFP fusion protein was examined further by fractionating HeLa cell lysates into cytosol and membrane fractions that were subsequently analyzed by Western blot using antibody against GFP. Unlike GFP, which is cytosolic, the majority of TE-GFP was detected in the membrane fraction (Fig. 5D), indicating that domain T is capable of targeting a heterologous protein to specific subcellular locations.

Analysis of the amino acid sequence of domain T failed to reveal any membrane-spanning structure. Therefore it is possible that the putative N-myristylation site is involved in membrane targeting. A mutation of glycine (+2) to alanine in domain T totally abolished membrane association (Fig. 5, C and D), and the mutant protein became exclusively cytosolic and indistinguishable from GFP (Fig. 5D). Because glycine (+2) has been shown to be essential for N-myristylation [26], it is likely that the mutation eliminated myristylation within domain T. To address this, HeLa cells, transfected with C3, C3tCAST, or mutant tCAST (C3tCAST G2A) were metabolically labeled with 9,10-[3H]myristic acid. Calpastatin proteins were immunoprecipitated using antibody directed against domain IV. The 3H-labeled tCAST was observed only in cells transfected with C3tCAST, indicating that it was myristylated. Substitution of glycine (+2) to alanine completely abolished myristylation (Fig. 6, top panel), while the synthesis of tCAST per
region from between human and mouse revealed a highly conserved surprising because tCAST contains a TATA-less promoter. The presence of multiple initiation sites is not considering 47 bp, with the major start site at 54 bp from the upstream from the start site in both human and mouse sequences. Sox5 and Sox6 are SRY-related proteins containing an HMG box [29, 30], and both are expressed in postmeiotic cells, suggesting that these factors might participate in the regulation of tCAST expression. Whether or not these putative binding sites are functional in the testis-specific expression of tCAST remains to be determined.

We have demonstrated that domain T is capable of targeting the heterologous protein GFP to the membrane. Fluorescence of the fusion protein appears to be concentrated in the cytoplasm with punctate and diffuse staining, indicative of this protein being associated with membranes of intracellular organelles. A similar punctate staining pattern has been detected on testis sections with discrete tCAST signals seen in round spermatids (data not shown). Although sCAST was found primarily in the cytosol in this study, other investigations suggested that sCAST could bind to membranes through electrostatic interaction between basic domain L and negatively charged phospholipids [31, 32]. This discrepancy with our results can be attributed to the tissue or cell-type specificity of sCAST localization or to the different methodology used in these experiments. While different members of the calpain family have been identified and found to localize to various subcellular sites [33], the predominant membrane localization of tCAST suggests that tCAST might exert its inhibitory effects on a subset of calpains that colocalize with tCAST. Consistent with this hypothesis, the majority of calpain activity in human sperm has been found recently to be membrane associated [34]. It has been shown that domain L can modulate the interaction between calpastatin and calpain I and II [35], and that lack of domain L and I alters the susceptibility of calpastatin to caspases [36]. Domain T of tCAST, in addition to its membrane targeting role, may also function by regulating specificity for different calpain isoforms. Dear et al. [37] recently characterized a testis-specific calpain 11 that shares 54.3% sequence similarity with calpain I. Although it is currently unclear which calpain isoforms are present in round spermatids, it remains of considerable interest to determine the specificity of tCAST to different calpains.

The myristic acid group serves as a hydrophobic membrane anchor [24], and our data show that myristylation is required for targeting tCAST to membranes. However, proteins bearing a myristic group are not always membrane bound. Various localizations have been reported for myristylated proteins, including the plasma membrane, cytosol, Golgi apparatus, endoplasmic reticulum, or nucleus, indicating that additional sequences and/or modifications may also regulate subcellular localization [38, 39]. Because tCAST was detected in both cytosol and membrane fractions in testis extract, we expect that additional levels of regulation are likely to contribute to the localization of tCAST and hence the modulation of its inhibitory activity. The observation of TE-GFP in both fractions indicates that these putative binding sites are functional in the testis-specific expression of tCAST.

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fuses, while only slight changes in protein levels of calpains were observed. A lower level of calpain activity caused by antisense oligonucleotides specific to calpains inhibits myoblast fusion. In contrast, downregulation of calpastatin protein using similar approaches accelerates the fusion processes [42, 43]. Because calpain has been localized to focal adhesions and is able to dynamically regulate cytoskeletal proteins and related proteins [44, 45], it is likely that the calpain-calpastatin system is also important for cell adhesion and migration [46, 47]. The synthesis of tCAST in round spermatids suggests that the protein may participate in spermiogenesis where round spermatids undergo extensive cell remodeling, subsequent sperm individualization, and release from Sertoli cells to become mature spermatozoa. Furthermore, the identification of calpain activity in the sperm indicates that tCAST may also function in sperm-egg fertilization processes, such as the acrosome reaction and gamete fusion. Further studies using gene targeting techniques to specifically knock out domain T of the calpastatin gene are expected to reveal physiological functions of tCAST during development.

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REFERENCES


