Biochemistry. In the article "Molecular cloning of a calmodulin-dependent protein phosphatase from murine testis: Identification of a developmentally expressed nonneural iso-enzyme" by Taro Muramatsu, Polavarapu Rathna Giri, Susumu Higuchi, and Randall L. Kincaid, which appeared in number 2, January 15, 1992, of Proc. Natl. Acad. Sci. USA (89, 529–533), the authors request that the following correction be noted. In preparing Fig. 3, on p. 531, the negative for the print in Fig. 3C was reversed, causing the order of the lanes to be backwards. This does not change the conclusion, i.e., that the three catalytic subunit genes show different hybridization patterns. The corrected figure and its legend are shown below.

![Corrected Figure 3](image)

Biochemistry. In the article "Prohormone processing in Xenopus oocytes: Characterization of cleavage signals and cleavage enzymes" by Judith Korner, Jay Chun, Laura O'Bryan, and Richard Axel, which appeared in number 24, December 15, 1991, of Proc. Natl. Acad. Sci. USA (88, 11393–11397), it is requested that the following correction be noted. In the last paragraph of page 11396, the statements, "In some precursors, the precise nature of the basic residue at P4 is also important. Mutation of the P4 arginine residue at the human immunodeficiency virus envelope processing site to lysine abolishes cleavage (48), whereas a similar mutation in the murine leukemia virus envelope glycoprotein has no effect on cleavage (49)" should be replaced by "In some precursors, the precise nature of the basic residue at P1 is also important. Mutation of the P1 arginine residue at the human immunodeficiency virus envelope processing site to lysine has no effect on cleavage (48), whereas a similar mutation in the murine leukemia virus envelope glycoprotein blocks cleavage (49)."

Biochemistry. In the article "An efficient method for generating proteins with altered enzymatic properties: Application to β-lactamase" by Arnold R. Oliphant and Kevin Struhl, which appeared in number 23, December 1989, of Proc. Natl. Acad. Sci. USA (86, 9094–9098), it is requested that the following be noted. In the abstract, line 7 should read "... selection. Here, a collection of 500,000 altered β-lactamase proteins..."

Biophysics. In the article "Conformational switching at cytochrome a during steady-state turnover of cytochrome c oxidase" by Robert A. Copeland, which appeared in number 16, August 15, 1991, of Proc. Natl. Acad. Sci. USA (88, 7281–7283), the following corrections should be noted. In the legends to Figs. 1 and 2, the sodium ascorbate concentrations should read 125 mM and 100 mM, respectively.
Molecular cloning of a calmodulin-dependent phosphatase from murine testis: Identification of a developmentally expressed nonneural isoenzyme
(calcineurin/spermatogenesis/phosphoproteins/gene diversity)

TARO MURAMATSU, POLAVARAPU RATNA GIRI, SUSUMU HIGUCHI*, AND RANDALL L. KINCAID

Section on Immunology, Laboratory of Molecular and Cellular Neurobiology, National Institute on Alcohol Abuse and Alcoholism, Rockville, MD 20852

Communicated by Martha Vaughan, October 4, 1991 (received for review August 21, 1991)

ABSTRACT A unique isofrom of the catalytic subunit of calmodulin-dependent protein phosphatase (CaM-Pp) was cloned from a murine testis library. The cDNA sequence of 1964 base pairs contained an open reading frame encoding a protein of 513 amino acids (Mr ~ 58,706), the predicted isoelectric point of which (pI 7.1) was much more basic than those of brain isoforms (pI 5.6–5.8). The deduced amino acid sequence was 77–81% identical to two other murine CaM-Pp genes and displayed a distinct Southern blot hybridization pattern, indicating that it was derived from a separate gene (type 3). High amounts of a 2800-nucleotide mRNA transcript were observed in testis, whereas mRNA species were not detectable in brain; thus, it seems likely that this CaM-Pp represents a nonneural isoenzyme. Measurements of CaM-Pp mRNA during testicular development showed a dramatic increase in expression during weeks 4–6, correlating with the later stages of spermatogenesis. These data suggest that this phosphatase isozyme may be involved in germ-cell function and are consistent with the report of a flagellum-associated form of CaM-Pp that may regulate sperm motility. [Tash, J. S., Krinks, M., Patel, J., Means, R. L., Klee, C. B. & Means, A. R. (1988) J. Cell Biol. 106, 1625–1633].

The regulation of phosphoprotein status by specific protein phosphatases is a fundamental control point in signaling pathways. A key question that remains unanswered concerns the mechanisms that provide for specificity in regulating the many cellular phosphoprotein substrates. For serine/threonine phosphatases, recent studies indicate that selectivity may be achieved through the use of various binding proteins that sequester catalytic subunits in different tissues or cellular compartments, and the existence of multiple isoforms of the catalytic subunits (for reviews, see refs. 1 and 2). For the calmodulin-dependent protein phosphatase (CaM-Pp), molecular cloning (3–8, 31) has demonstrated two distinct genes (type 1 or “α” and type 2 or “β”) for the 60-kDa catalytic subunit, each of which can undergo alternative splicing to yield additional variants (4, 5).

The biological roles of isoforms of the Ca2+-/calmodulin (CaM)-regulated phosphatase are poorly understood. The high amounts of the brain-specific “calcineurin” (9) isoenzymes in the central nervous system suggest a role in the development and maintenance of neuronal phenotype, or in neurotransmission, per se. Other lines of evidence suggest that this phosphatase may regulate membrane conductance through effects on voltage-gated ion channels (10, 11). In skeletal muscle, a form of this enzyme, protein phosphatase 2B (PrP-2B), plays a physiological role in counteracting the epinephrine-induced breakdown of glycogen that is mediated by cAMP-dependent protein kinase (12). In this case, the major substrate of the Ca2+-dependent phosphatase is the phosphoprotein inhibitor of protein phosphatase 1 (PrP-1); thus, PrP-2B can act as a signal amplifier to increase the dephosphorylation carried out by other phosphatases. In lymphocytes and in brain, evidence exists for CaM-Pp forms that are associated with the cytoskeleton and/or the plasma membrane, as well as for cytosolic forms (13–16); such findings may suggest a role in regulating cellular ultrastructure. Also, recent studies on the biochemistry and cell biology of sperm described a form of CaM-Pp that was physically associated with the flagellum (17). It was proposed that this phosphatase might alter motility by regulating the phosphorylation of axokinin, the major cAMP-dependent phosphoprotein present on this organelle.

As reported here, we have characterized a unique form of the CaM-Pp catalytic subunit that appears to be specifically expressed in testis. Its structure indicates that it is derived from a distinct mammalian gene (type 3) that is homologous to the neuronal isoforms recently identified. Importantly, its developmental expression in testis suggests that this nonneural isoenzyme may play a role in germ-cell function.

METHODS

Materials. All restriction enzymes and the Klenow fragment of DNA polymerase I were purchased from Promega or from United States Biochemical. Reagents for DNA sequencing, using a modified form of T7 DNA polymerase (Sequenase), were from United States Biochemical. Components used for the PCR were obtained from Perkin–Elmer/Cetus and oligonucleotide primers were synthesized with a Cyclone Plus DNA synthesizer (MilliGen/Biosearch, Novato, CA). Hybrid nylon membranes used for the screening of phage libraries were from Bios (New Haven, CT), and filters used for Southern and Northern blots were from DuPont. [32P]dATP and [32P]dCTP were purchased from DuPont/NEN. All materials for electrophoresis were obtained from Bio-Rad.

Isolation and Characterization of cDNA Clones. The template for making a hybridization probe was generated by PCR amplification of a 94-base-pair (bp) region of a partial murine thymocyte clone (TC1.2) that was 78% identical to bp 1059–1149 of CNα-4, a murine brain clone (4). Because a 103-bp region in TC1.2, adjacent to this, was 92% identical to bp 955–1058 of CNα-4, the 94-bp region was selected to minimize potential cross-hybridization. The cDNA was amplified using a sense-strand primer, 5’-CTTTGCGCCTTTTGTTG-

Abbreviations: CaM, calmodulin; CaM-Pp, CaM-dependent protein phosphatase; PrP-1, protein phosphatase 1; PrP-2B, protein phosphatase 2B; UTR, untranslated region.
*Present address: Japanese National Institute on Alcoholism, Kurihama National Hospital, Yokosuka-shi 239 Japan.
†The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession no. M81475).

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GAGA-3', and a primer from the antisense-strand, 5'-ACCTTCTTTCATCGGTGA-3'. After gel purification of the PCR fragment as described (4), a radiolabeled probe was prepared by primer extension of this template with the Klenow fragment of DNA polymerase I using [32P]dCTP and [32P]dATP (3000 Ci/ml; 1 Ci = 37 GBq) and the same two primers. When Northern blot analysis was carried out, this probe bound selectively to testis mRNA but not to brain mRNA. Therefore, it was used to screen a mouse testis cDNA library constructed in Uni-ZAP XR vector (Stratagene) by plaque hybridization. After denaturation, the plaque-containing filters (~100,000 plaque-forming units per 150-mm dish) were incubated for 2–4 hr at 42°C in prehybridization solution [4X standard saline citrate (SSC)/5X Denhardt's solution/0.2% SDS/yeast tRNA (50 µg/ml)/herring sperm DNA (100 µg/ml)/50% (vol/vol) deionized formamide]. Hybridization was carried out for 12–16 hr at 42°C in the same solution containing 10% (wt/vol) dextran sulfate and 1 × 10^6 cpm of probe per ml of hybridization solution. The filters then were washed sequentially for 30-min periods with 2 × SSC/0.1% SDS, 0.2 × SSC/0.1% SDS, and 0.1 × SSC/0.1% SDS at room temperature. Filters were exposed to Kodak XAR-5 film overnight at ~70°C with intensifying screens. After plating of the initial positive clones at a density of ~300 plaque-forming units per dish, individual plaques were excised and extracted with 1 ml of 10 mM Tris-HCl, pH 7.8/10 mM MgCl2. The insert-containing portions of phage DNA from positive clones were excised in vivo as phagemids in Escherichia coli and purified using the alkaline lysis procedure (18). DNA sequence was determined by the dideoxynucleotide chain-termination method (19). The sequencing reactions for the longest clone (MTα-1) were done twice on both strands using primers located ~200 bp apart (Fig. 1).

**Southern and Northern Blot Analysis.** Hybridization probes were generated by PCR amplification of a homologous 122-bp region (see Fig. 1) in three murine cDNAs, each representing a distinct gene for the catalytic subunit; this corresponds to bp 284–405 of CNα-4 (gene 1, ref. 4), bp 280–401 of TCα-3.
information from the image is too fragmented to be accurately transcribed.
independent genes for the CaM-PrP catalytic subunit are present in mammals and that the testis cDNA is not a cloning artifact due to contamination of a library with nonmammalian cDNAs (25).

Expression of mRNA for the Testis-Specific CaM-PrP. Preliminary experiments indicated that the expression of the type 3 gene in brain was negligible when RNA from whole brain was compared to an equal amount of testis RNA (data not shown). To see whether small amounts of transcript might be concentrated in specific brain regions, RNAs isolated from hippocampus, cerebellum, cerebral cortex, and midbrain were compared along with those from skeletal muscle, ovaries, and testis by using homologous probes for the three genes (Fig. 4). Although the distribution of type 1 mRNAs [4.0 and 3.6 kilobases (kb), Fig. 4B] and type 2 mRNA (3.4 kb, Fig. 4C) was similar in different brain regions, the 2.8-kb type 3 transcript was undetectable (Fig. 4D). Furthermore, very little type 3 mRNA was observed in other nonneural tissues (Fig. 4 and unpublished data), underscoring its highly specific expression in testis.

Developmental studies of this CaM-PrP isoform showed very little mRNA expression until day 20 postpartum, at which time the amounts increased dramatically, peaking on day 40 (Fig. 5). Throughout this period there was no evidence of transcripts of different sizes, suggesting that no major changes in the processing of mRNA (e.g., alternative splicing) occurred during this time. The increased amounts of mRNA parallel hormonal and functional changes during spermatogenesis and suggest that expression of this isoenzyme may accompany the maturation of germ cells.

**DISCUSSION**

A considerable amount is known about the properties and localization of calcineurin, the brain-specific form of CaM-PrP. This abundant CaM-binding protein is apparently ubiquitous in neurons and has been proposed to be a neuronotypic marker (26). However, several studies suggested that nonneural tissues contained distinct isoforms of this phosphatase because, although the intrinsic calcium-binding (B or "β") subunit was detected immunologically, the catalytic (A or "α") subunit appeared to be recognized poorly by antibodies raised against the brain enzyme (27, 28). The present study supports those observations by characterizing a cDNA that presumably encodes a nonneural isoform of CaM-PrP in testis, although the final proof of this must await expression of its enzyme activity. We had suggested (4) such a related gene product in testis based on paradoxical Northern blot results obtained with probes for a murine brain cDNA (type 1 gene). In that study, we reported (4) that the predominant 2.8-kb mRNA in testis hybridized relatively poorly when probes representing only the 5' half of the open reading frame

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**Fig. 4.** Comparison of mRNA expression in various tissues by using probes for three murine CaM-PrP genes. Total RNA was prepared from different brain regions and tissues of an adult mouse, and 4--6 μg of each sample was electrophoresed in a 1% agarose gel containing formaldehyde. After transfer to GeneScreen nylon membrane (DuPont/NEN), the blot was hybridized sequentially with 32P-labeled antisense probes. RNA samples were from hippocampus (lane 1), cerebellum (lane 2), cerebral cortex (lane 3), midbrain (lane 4), muscle (lane 5), ovary (lane 6), and testis (lane 7). (A) Ethidium bromide-stained RNA gel. (B--D) Autoradiograms of Northern blot after hybridizing to probes for genes 1--3, respectively; the positions of 28S and 18S rRNAs are indicated by tick marks.

**Fig. 5.** Developmental expression of CaM-PrP catalytic subunit mRNA in testis. Total testicular RNA was prepared from mice of various ages and a Northern blot containing 4--6 μg of each sample was prepared as described in Fig. 4. Samples were from 10-day (lane 1), 15-day (lane 2), 20-day (lane 3), 30-day (lane 4), 40-day (lane 5), and adult (lane 6) animals. (A) Ethidium bromide-stained blot after transfer of RNA. (B) Autoradiogram of blot hybridized to antisense probe for gene 3. (C) Quantitative analysis of data. The amounts of radioactivity in the bands shown in B were quantified directly using a radioimaging device (Betascope 603, Betagen, Waltham, MA); these were plotted after adjusting for the amount of RNA loaded in each lane. Positions of 28S and 18S RNA are indicated by tick marks in A and B.
were used instead of the full-length probe. That result apparently reflected a lesser degree of nucleotide sequence identity in the 5' halves than in the 3' halves of the type 1 and type 3 genes.

One striking feature in the overall deduced properties of the testis form is that its isoelectric point (pI 7.1) is much higher than those of the brain isoforms (pI 5.6–5.8). Because the catalytic, CaM-binding, and autoinhibitory domains have been highly conserved, this difference apparently results from basic residues in the nonconserved regions of the peptide, especially those near the carboxyl terminus. This positively charged area, which is also rich in histidine, may be a site for regulation (e.g., by phosphorylation) or may impart some other distinctive feature(s) (e.g., cellular localization, metal dependency, or substrate specificity) to this isoenzyme.

Perhaps the most provocative finding of the present study is that increases in CaM-PrP mRNA expression correlate with hormonally regulated stages in spermatogenesis. In C57BL mice, serum levels of the gonadotropins, luteinizing hormone, and follicle-stimulating hormone increase sharply between days 20 and 35 (29, 30), precisely when CaM-PrP mRNA expression increases. During this period, immature spermatids become elongated, and motile spermatozoa are present by week 6. The correspondence of these events with increased CaM-PrP mRNA levels suggests that this enzyme may play a role in germ-cell maturation. It is tempting to speculate that this isoenzyme corresponds to the form, reported by Tash et al. (17), that cosediments with the dynein ATPase of isolated sperm flagella. If so, it should be possible to test the hypothesis that this isoenzyme is physiologically important in regulating motility by using site-directed molecular and immunological reagents and through production of transgenic animals. The developmental regulation of this CaM-PrP isom form may provide a useful model for examining molecular aspects of gene expression and, perhaps, yield insights into the role of Ca²⁺-regulated dephosphorylation in testicular maturation.

Note Added in Proof. While this paper was in press, a rat testis cDNA for a "calcineurin B-like protein" was described (32); its tissue-specific expression may suggest that it is part of an isoenzyme containing the type 3 catalytic subunit reported here.