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STRUCTURE AND EXPRESSION OF TWO ISOFORMS OF THE MURINE CALMODULIN-DEPENDENT PROTEIN PHOSPHATASE REGULATORY SUBUNIT (CALCINEURIN B)

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SUMMARY: Murine cDNAs representing distinct genes for the regulatory subunits of calmodulin-dependent protein phosphatase (CaM-PrP) were cloned from a testis library, using probes prepared by PCR amplification of brain and testis mRNA. The cDNA sequence of the brain-specific isoform (β1) encodes a 170 amino acid protein (M_r ≈19.3 kDa), whereas that for the testis isoform (β2) contains 179 residues (M_r ≈20.7 kDa); these two sequences show ≈80% amino acid identity. An oligonucleotide probe for the brain isoform hybridized to a single mRNA of 3.6 kilobases (kb) in many tissues, whereas using the β2 probe, two mRNAs of 1.8 and 0.8 kb were detected only in testis. The mRNA for the testis-specific isoform increases markedly during development, its pattern being virtually identical to that of mRNA for a testicular form of the catalytic subunit (α3). These data are consistent with the biological co-regulation of catalytic and regulatory subunits of a testis-specific isoenzyme during germ cell maturation.

Calmodulin-dependent protein phosphatase (PP2B, calcineurin) may play an important role in neural and nonneural Ca^{2+}-regulated signaling events, including those involved in muscle glycogen metabolism (1), ion channel regulation (2), sperm motility (3) and lymphocyte function (4). Recent findings showing that this enzyme is a cellular target for certain immunosuppressive drugs have implicated this enzyme in the control of T-cell proliferation (5-7). The enzyme is a heterodimer of a 60 kDa catalytic subunit and an intrinsic 19 kDa regulatory subunit that is homologous to other Ca^{2+} binding proteins (8). For the 60 kDa catalytic subunit, molecular cloning has demonstrated three distinct mammalian genes that can undergo alternative splicing to yield additional variants (9-15).

Recently, two groups reported independently the molecular cloning of a testis-specific homolog of the regulatory B subunit in rat (16,17). In this report, two isoforms of the regulatory subunit were identified in a murine testis library, the genes for which we designate *PP2Bβ1* and *PP2Bβ2*. The distribution of mRNA for these B subunit isoforms in various tissues suggests that there may be two major classes of heterodimeric isoenzyme, one that is present in many

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tissues but having highest concentrations in brain (neural type), and another that is greatly enriched in testis (testicular type). Further, comparison of the mRNA expression for testis-specific catalytic and regulatory subunits suggests that their production may be closely linked during germ cell development.

MATERIAL AND METHODS

Materials: Reagents for DNA sequencing, using the modified form of T7 DNA polymerase (Sequenase) were purchased from United States Biochemical, as was T4 DNA ligase. The Klenow fragment of DNA polymerase I and reagents for reverse transcriptase reactions were from United States Biochemical and Promega. All components used for the PCR amplification were obtained from Perkin-Elmer/Cetus and oligonucleotide primers were synthesized with a Cyclone Plus DNA synthesizer (Milligen/Biosearch, Novato, CA). Hybond nylon membranes used for the screening of phage libraries were from Amersham Corp., and Gene-Screen filters used for Northern blots were from Dupont. [^{32}P]dATP, [^{32}P]dCTP and [^{35}S]dATP were purchased from Dupont/NEN. All materials for electrophoresis were obtained from Bio-Rad.

Cloning of the murine regulatory subunits: Tissue dissected from mouse testis and various brain regions was immediately frozen in liquid nitrogen and stored at -70°C prior to use. Total RNA was isolated from frozen tissues by guanidine isothiocyanate extraction followed by precipitation with LiCl. Polyadenylated mRNA was purified from total RNA using a poly(A) Quik mRNA purification column (Stratagene), and subjected to first-strand cDNA synthesis according to the manufacturer's protocol. First-strand cDNA, derived from brain (hippocampus) mRNA, served as the template for PCR amplification using "sense" and "anti-sense" primers based on the published cDNA sequence for the human brain B subunit (18), 5'-AGCCAGCGAGCCGCCGAC-3' and 5'-TTGGGTGGTACTCTCTGA-3'. Using this PCR product as template, a second round of PCR ("nested PCR") was done using two new primers, 5'-CGACCCGCCGAGCAAAAT-3' and 5'-GATAAGAGTCACACATCT-3'. Similarly, cDNA made from mouse testis mRNA was used as the template for the PCR amplification of a testis-specific isoform of the regulatory subunit, based on the sequence reported from rat testis (16). Primers for the first round of PCR were 5'-CCACTGCCTCGCCAT-3' and 5'-CAGATGTACACCTCTC-3'; for nested PCR, these were 5'-ATGGGAAATGAGGCAAGT-3' and 5'-GCTTTTAGTCTTCTTG-3'. All PCR products were analyzed on 1% agarose gels. DNA fragments of the expected sizes were purified by polyacrylamide gel electrophoresis, and then inserted into the Sma I site of pUC-18 plasmid using T4 ligase. This step, as well as the purification of plasmid DNA using the alkaline lysis procedure, was carried out as described (9).

After verifying the authenticity of PCR fragments by DNA sequencing, probes were generated by PCR amplification of these plasmids using primers encompassing the open reading frame (ORF) of each mouse regulatory subunit, i.e., for β1, primers 5'-ATGGGAAATGAGGCAAGT-3' and 5'-GATAAGAGTCACACATCT-3' and for β2, primers 5'-ATGGGAAATGAGGCAAGT-3' and 5'-GCTTTTAGTCTTCTTG-3'. For the brain isoform, a biotinylated probe was made by random primed labeling of the DNA fragment (19), and for the testis form, a radiolabeled probe was prepared by primer extension of the PCR fragment with the appropriate sense and antisense primers. A mouse testis cDNA library constructed in a Uni-ZAP XR vector (Stratagene) was screened by plaque hybridization as previously described (14). 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. DNA sequence, determined by the dideoxynucleotide chain-termination method (20), was confirmed on both strands using sequencing primers located 150-200 bp apart.

RNA preparation and Northern blot analysis: Total RNA from various mouse tissues was extracted according to the procedure of Cathala *et al.* (21) and prepared for Northern blots as described (12). Hybridization probes were generated by PCR

amplification of regions within the ORF of each regulatory subunit isoform. For the brain-specific form this corresponds to bp 96-504, whereas for the testis subunit this corresponds to bp 100-391. The nucleotide sequence of these two areas differ from each other by at least 25%; this prevents cross-hybridization of probes under the conditions of stringency used in this study. Radiolabeled hybridization probes were prepared by primer extension of these templates with specific anti-sense primers as described (12). Northern blots were incubated for at least 2 hr at 42°C in prehybridization solution prior to hybridization for 12-16 hr (42°C) in the same solution containing 10% dextran sulfate and $1x10^6$ cpm of the probe per ml of solution. The blots were then washed sequentially for 15 min periods with 2X SSC/0.1%SDS and 0.2X SSC/0.1%SDS at room temperature, following by a final wash at 55°C in 0.1X SSC/0.1%SDS. The blots were exposed to Kodak XAR-5 film at -70° with intensifying screens.

RESULTS AND DISCUSSION

Structural comparison of brain and testis regulatory subunits

Two cDNAs encoding distinct isoforms of the CaM-PrP regulatory subunit (calcineurin B) were identified using nested PCR amplification of brain and testis mRNA, as outlined under Methods. Probes based on these DNA fragments were used to screen a murine testis library prepared using germ cell-enriched mRNA. Out of 240,000 plaques screened with a biotinylated probe for the brain (β1) form, 1 positive clone was found, the sequence of which was identical to the COOH-terminal 117 amino acids specified by the PCR fragment, plus approximately 500 bp of 3' untranslated region (UTR) (Fig. 1). The ORF of the β1 form encodes a protein of 170 amino acid with a calculated molecular mass of 19.3 kDa. As for the testis (β2) isoform, 3 clones were found out of 360,000 plaques screened with a radiolabeled testis probe. All of these contain the identical ORF of 540 bp, preceded by 66 bp of 5' UTR and followed by 178 bp of 3' UTR that includes a poly(A) tail. The deduced sequence encodes a protein with a molecular mass of 20.7 kDa comprised of 179 amino acids. Both regulatory subunits show four conserved Ca^{2+} binding domains and have the same predicted isoelectric point (4.5), suggesting their functional equivalence. However, the deduced sequences of the two murine regulatory subunits are only 80.4% identical, with the greatest difference in the NH_2-terminal third of the ORF (Fig. 2). The deduced sequence of the β1 isoform shows only two differences with that of human brain B subunit (20), one of which (pos. 3, asn vs. ser) is present in the consensus site for myristoylation. On the other hand, the deduced sequences of PP2Bβ2 are only 89% identical in the two rodent species (rat and mouse). Also, the murine β2 isoform has three additional amino acids, compared with rat, resulting from the insertion of an additional nucleotide in the rat stop codon. These findings appear to indicate a reduced evolutionary pressure on the structure of *PP2Bβ2*, especially in comparison to the highly conserved *PP2Bβ1*.

Differential expression of mRNAs for *PP2Bβ1* and *PP2Bβ2*

Northern blot analysis of the two isoforms shows a 3.6 kb *PP2Bβ1* transcript that is expressed very highly in brain and at reasonable levels in many tissues,

A

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ATGGGAAGTGAGGCGAGTTACCCTTTGGAA ATGTGCTCACACTTTGATGCTGATGAAATT AAAAGGCTAGGAAAGAGATTCAAGAAGCTT GATTTGGACAATTCTGGTTCTTTGAGCGTG 120
M G S E A S Y P L E  M C S H F D A D E I  K R L G K R F K K L  D L D N S G S L S V  40

GAAGAGTTCATGTCTCTGCCTGAGTTACAG CAGAACCCTTTAGTACAGCGGGTAATAGAT ATATTCGACACAGACGGCAACGGAGAAGTG GACTTCAAAGAATTCATTGAAGGAGTGTCT 240
E E F M S L P E L Q  Q N P L V Q R V I D  I F D T D G N G E V  D F K E F I E G V S  80

CAGTTCAGTGTCAAAGGCGATAAGGAACAG AAGTTGAGGTTTGCTTTTCGTATCTATGAC ATGGATAAAGATGGCTATATTTCCAATGGA GAACTCTTCCAGGTGTTGAAGATGATGGTG 360
Q F S V K G D K E Q  K L R F A F R I Y D  M D K D G Y I S N G  E L F Q V L K M M V  120

GGCAACAATCTGAAAGATACACAATTACAG CAGATTGTAGACAAAACCATAATAAATGCA GATAAGGATGGAGATGGAAGAATATCCTTT GAGGAATTCTGTGCTGTCGTAGGTGGCCTA 480
G N N L K D T Q L Q  Q I V D K T I I N A  D K D G D G R I S F  E E F C A V V G G L  160

GATATCCACAAAAAGATGGTGGTGGATGTG TGACTCTTTGAAGGAATACCACCCAACACT TTTGCTTTCTTCTCCATCTCTGAAGATCTG CTCAAGACGTCAGCATGCTCTCTGTGTATT 600
D I H K K M V V D V  . 170
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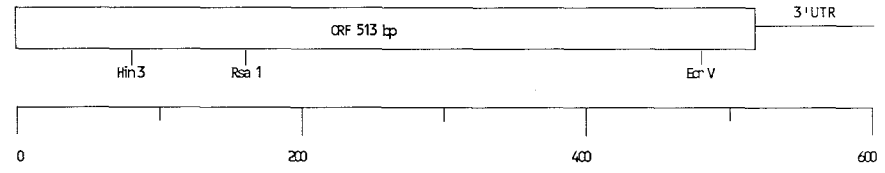

B

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                                                                -66  GCAGGA ATTCGGCACGAGGTTTGCCCTGCCGTCTCC TCCCTCCTTTGCCAGCCCTACCGCCTCGCC  -1
ATGGGAAATGAGGCCAGCTACCAAACTGAG CTGTGCAACCACTTCGACCAGGAAGAGATA AGAAGGCTGGGTAAAAGCTTCAGGAAGCTG GACTTGGACAAATCGGGCTCCCTGAGCATA 120
M G N E A S Y Q T E  L C N H F D Q E E I  R R L G K S F R K L  D L D K S G S L S I  40

GAAGAGTTCATGAGGCTGCCTGAGCTGCAG CAGAATCCGCTGGTGGGCCGAGTGATCGAC ATCTTCGACACAGACGGCAACGGGGAAGTG GACTTCCACGAGTTCATCGTGGGCACCTCC 240
E E F M R L P E L Q  Q N P L V G R V I D  I F D T D G N G E V  D F H E F I V G T S  80

CAGTTCAGCGTCAAGGGTGATGAAGAGCAG AAGCTAAGGTTCGCCTTCAGAATCTACGAC ATGGATAATGATGGCTTCATCTCCAATGGG GAGCTCTTCCAGGTGCTTAAGATGATGGTG 360
Q F S V K G D E E Q  K L R F A F R I Y D  M D N D G F I S N G  E L F Q V L K M M V  120

GGAAACAACCTCAAGGACTGGCAGCTGCAG CAGCTGGTGGACAAGAGCATCTTGGTCCTG GATAAGGATGGCGATGGCCGGATATCCTTT GAAGAGTTCAGTGATGTGGTCAAGACCATG 480
G N N L K D W Q L Q  Q L V D K S I L V L  D K D G D G R I S F  E E F S D V V K T M  160

GAGATCCACAAGAAATTGGTCGTGTTTGTA GAACACGGTCAAGAAGACTTAAAAGCTTAA AACTAAGAAAATGCACATCTTTGCCTTTCT TTCTCAAGCTTGTGTATGTGGGAGAAGACA 600
E I H K K L V V F V  E H G Q E D L K A .  179

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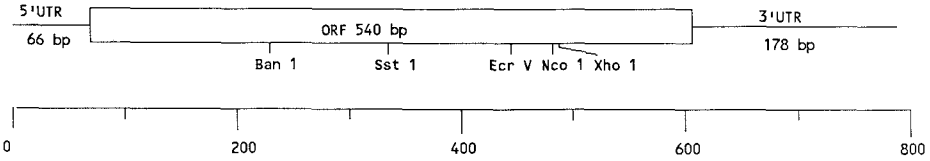

Fig. 1. Nucleotide and deduced amino acid sequences of mouse brain (β1) and testis (β2) isoforms of the CaM-PrP regulatory subunit. Panel A; The cDNA sequence for *Pp2bβ1* is a composite of sequence obtained by PCR amplification of brain mRNA (see Methods) and a testis clone that contains a region of additional 3' untranslated sequence (UTR). Approximately 300 bp of 3' UTR, omitted from the figure to conserve space, has been submitted to GenBank. Panel B; The cDNA for the testis isoform (*Pp2bβ2*), reflecting a full-length clone isolated from a testis library. Maps of unique restriction sites within the ORF for each regulatory subunit isoform are shown at the bottom of each panel.

such as muscle, thymus and spleen. In contrast, mRNAs of 1.8 and 0.8 kb for *PP2Bβ2* are seen only in testis and are essentially undetectable in other tissues (Fig. 3). These data are in agreement with those reported in the rat, (16,17)

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PP2Bβ1   MGSEASYPLEMCSHFDADEIKRLGKRFKKLDLDNSGSLSVEEFMSLPELQQNPLVQRVID  60
PP2Bβ2   ..N....QT.L.N...QE..R....S.R.....K.....I....R..........G....  60

PP2Bβ1   IFDTDGNGEVDFKEFIEGVSQFSVKGDKEQKLRFAFRIYDMDKDGYISNGELFQVLKMMV  120
PP2Bβ2   ............H...V.T........E..............N..F.............  120

PP2Bβ1   GNNLKDTQLQQIVDKTIINADKDGDGRISFEEFCAVVGGLDIHKKMVVDV*          170
PP2Bβ2   ......W....L...S.LVL..............SD..KTME....L..F.EHGQEDLKA*  179
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Fig. 2. Comparison of the deduced amino acid sequences of PP2Bβ1 and PP2Bβ2, encoding murine brain- and testis-specific regulatory subunits. The cumulative number of residues for each isoforms is indicated on the right. Positions of identity between the two sequences are indicated by periods.

although the sizes of the murine testis-specific mRNAs appear to be much smaller. The tissue distribution of transcripts parallels the pattern of expression seen for murine brain (α1, α2) and testis (α3) catalytic subunit genes (12,14); this may suggest that the neural catalytic subunits form complexes only with the brain-specific (β1) regulatory subunit whereas the testis enzyme would be a heterodimer of α3 and β2 subunit isoforms. However, mRNA for the brain-specific isoform is observed in testis (Fig. 3), consistent with data reported in the rat (16) and very small amounts of both α3 and β2 are found in most tissues when Northern blots are exposed for 8-12 days. Thus, it is possible that "hybrid" holoenzymes containing different combinations of these catalytic and regulatory subunits exist in specific tissues and cell types.

Expression of mRNAs in developing mouse testis

In testis, expression of mRNA for the catalytic subunit of CaM-PrP (*PP2Bα3*) is strongly correlated with hormonally-regulated stages in spermatogenesis (14); this suggests a role in the maturation or function of germ cells. When the same Northern blot was hybridized to probes for the two regulatory subunits, only the β2 mRNA was clearly detected (Fig.4). After quantifying the radioactivity in the bands, the pattern appears almost identical to that seen for the catalytic

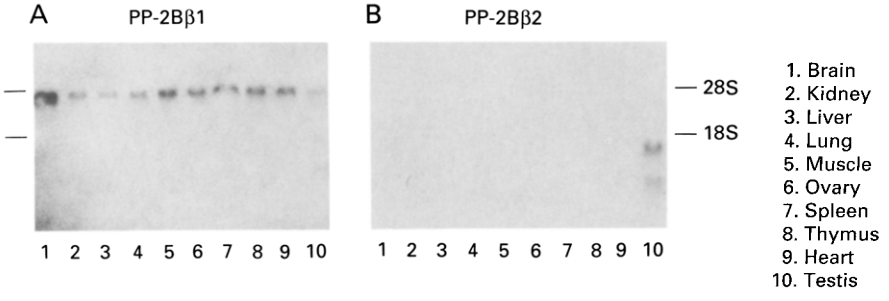

Fig. 3. Expression of mRNA in various murine tissues for β1 and β2 isoforms of the CaM-PrP regulatory subunit. Total RNA was prepared from different tissues of adult mice and portions of these samples were electrophoresed in a 1% agarose gel containing formaldehyde. Lane 1 contains 5 μg of brain RNA and lane 10 contains 20 μg of testis RNA; the remaining lanes contain ≈ 10 μg each of RNA from other tissues, as indicated. After transfer of RNA to the nylon membrane, the blot was hybridized sequentially with ^{32}P-labeled anti-sense probes. Autoradiographs of Northern blot after hybridizing to probes for *PP2Bβ1* (panel A) and *PP2Bβ2* (panel B). Positions of 28S and 18S rRNA are indicated.

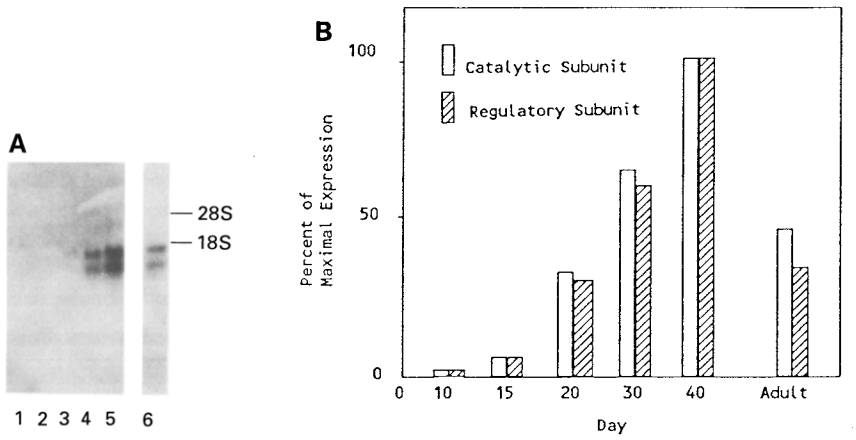

Fig. 4. Developmental expression of the β2 regulatory subunit mRNA in mouse testis. Panel A. Total testicular RNA was prepared from mice of various ages and a Northern blot was prepared as described (14). Lanes 1-5 contain 4-6 μg of total testis RNA from 10, 15, 20, 30 and 40 day old mice; that in lane 6 is from an adult mouse. The blot was hybridized with an anti-sense probe for *PP2Bβ2*, as described in the legend to Fig. 3. Positions of 28S and 18S rRNAs are indicated by tick marks. Panel B. Quantitative analysis of mRNA hybridization for the testis-specific forms of the catalytic (PP2Bα3) and regulatory subunits (PP2Bβ2) subunits. Data from ref. 14 and that presented here were collected using a radioimaging device (Betascope 603, Betagen Corp., Waltham, MA) and normalized to a value of 100% for peak expression (day 40). These relative expression values were plotted after correction for background and for different RNA loadings.

subunit. These data, which are similar to those for β2 expression in rat (17), argue for a hormonal co-regulation of regulatory and catalytic subunits during testis maturation. Future studies on the expression of the subunits in isolated cellular populations within the testis (*e.g.*, germ cells, Sertoli cells, etc.) will be needed to determine the biological significance of these observations.

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