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Differential Expression of Protein Phosphatase 1 Isoforms in Mammalian Brain

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Rat cDNAs encoding neuronal isoforms of protein phosphatase 1 (PP1) were isolated and their primary structures elucidated. The derived amino acid sequences allowed us to design synthetic C-terminal peptides that were used to raise antibodies. Isoform-specific anti-peptide antibodies against PP1α and PP1γ1 were used to investigate the tissue distribution of PP1 isoforms by immunoblotting. Both isoforms were ubiquitously expressed in mammalian tissues, with the highest levels being observed in brain. Of all neuronal tissues examined, PP1α and PP1γ1 were found to be most abundantly expressed in the striatum. Lesion experiments with kainic acid indicated that both the α and the γ1 isoforms of protein phosphatase 1 were relatively enriched in the medium-size spiny neurons of the striatum. "*In situ*" hybridization to rat brain slices using highly sensitive riboprobes also showed PP1α, PP1β, and PP1γ1 to be widely expressed in mammalian brain. However, some interesting differences were observed. For example, PP1α and PP1γ1 were found to be expressed in the striatum, where DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, M_r = 32,000 Da) is also known to be highly expressed. PP1β appeared to be relatively less abundant in the same cells, as judged both by "*in situ*" hybridization and by the apparent absence of PP1β clones from the striatal cDNA libraries used.

[Key words: DARPP-32, in situ, kainic acid, phosphatase, PP1, striatum]

The balance between protein kinase and phosphatase activities in eukaryotic cells is responsible for controlling levels of protein phosphorylation, and is thought to be the major regulatory mechanism governing a variety of cellular processes (Nestler and Greengard, 1984; Hunter, 1987). Indeed, it is likely to be of particular importance in mammalian brain since kinases and phosphatases are particularly abundant in this tissue (Nairn et al., 1985; Shenolikar and Nairn, 1991). The family of serine/threonine-specific protein phosphatases was originally divided into four groups (types 1, 2A, 2B, and 2C), according to substrate specificity and metal ion dependence (Ingebritsen and Cohen, 1983). However, recent work has indicated that whereas phosphatases type 1, 2A, and 2B are highly homologous members of the same family (Berndt et al., 1987; Shenolikar and Nairn, 1991), phosphatase 2C seems to belong to an unrelated family (Tamura et al., 1989). PP1 (protein phosphatase 1) is known to be widely distributed in mammalian tissues and to have a broad substrate specificity *in vitro*. It is thought to be regulated *in vivo* by its interaction with regulatory and inhibitory proteins (Shenolikar and Nairn, 1991). These proteins can be phosphorylated/dephosphorylated in response to extracellular stimuli and thus dynamically regulate the activity of PP1.

At least three different heat-stable inhibitor phosphoproteins of protein phosphatase 1 are known to exist in mammalian cells [inhibitor 1, inhibitor 2, and DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, M_r = 32,000 Da)]. Inhibitor 1 and DARPP-32 share many biochemical similarities, including primary structure homology, heat and acid stability, and the ability to potently inhibit PP1 following phosphorylation by cAMP-dependent protein kinase (Hemmings et al., 1984a,b; Williams et al., 1986). Although structurally and functionally related, inhibitor 1 and DARPP-32 exhibit different patterns of expression. Whereas inhibitor 1 is ubiquitously expressed in a variety of mammalian tissues, DARPP-32 expression is relatively restricted, being most abundant in the medium-sized spiny neurons of the striatum. Furthermore, recent studies indicate that both are relatively enriched in striatal neurons and striatonigral fibers (Nairn et al., 1988). Given the central role of reversible protein phosphorylation in the control of neuronal function, it is important to determine the distribution of protein phosphatases in mammalian brain. In the present study we have investigated the distribution of various isoforms of protein phosphatase 1 in the brain.

Some of the data presented here was the subject of a preliminary report (da Cruz e Silva and Greengard, 1992).

Materials and Methods

Materials. Hybond C membranes, anti-tubulin, anti-actin, and anti-glial fibrillary acidic protein (GFAP) antibodies were obtained from Amersham (Arlington Heights, IL). Restriction enzymes and T4 polynucleotide kinase were obtained from GIBCO–Bethesda Research Labs (Gaithersburg, MD). Millex GV filters were purchased from Millipore Corporation (Bedford, MA). Oligonucleotides were from Operon Technologies, Inc. (Alameda, CA). Ficoll 400, Sephadex G-25, and activated CH Sepharose 4B were supplied by Pharmacia LKB Biotechnology

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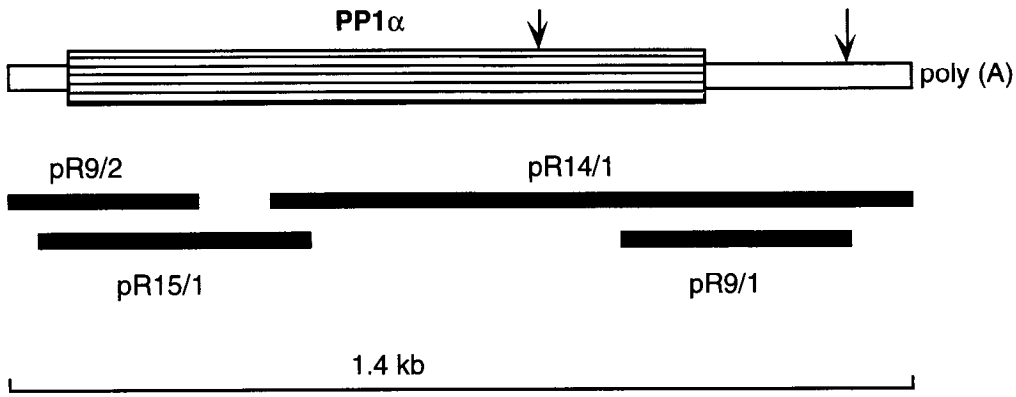
A)



B)

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  87 ATGTCCGACAGCGAGAAGCTCAACCTGGAT TCCATCATCGGGCGCCTGCTGGAAGTGCAG GGCTCACGGCCTGGAAAGAATGTGCAGCTG
   1  M  S  D  S  E  K  L  N  L  D   S  I  I  G  R  L  L  E  V  Q   G  S  R  P  G  K  N  V  Q  L

 177 ACAGAGAACGAGATCCGTGGTCTTTGCCTC AAATCCCGGGAGATTTTCCTGAGCCAGCCT ATTCTTCTGGAGCTTGAGGCGCCTCTCAAG
  31  T  E  N  E  I  R  G  L  C  L   K  S  R  E  I  F  L  S  Q  P   I  L  L  E  L  E  A  P  L  K

 267 ATCTGTGGTGACATCCATGGCCAGTACTAT GACCTTCTACGGCTGTTCGAGTATGGTGGC TTCCCTCCAGAGAGCAACTACCTCTTCTTG
  61  I  C  G  D  I  H  G  Q  Y  Y   D  L  L  R  L  F  E  Y  G  G   F  P  P  E  S  N  Y  L  F  L

 357 GGGGACTATGTGGATCGGGGCAAGCAGTCT TTGGAGACCATCTGCTTGTTGCTGGCCTAT AAGATCAAATACCCGGAGAATTTCTTTCTA
  91  G  D  Y  V  D  R  G  K  Q  S   L  E  T  I  C  L  L  L  A  Y   K  I  K  Y  P  E  N  F  F  L

 447 CTACGTGGGAACCATGAGTGTGCCAGCATC AACCGCATCTATGGCTTCTATGATGAATGC AAGAGACGATACAACATCAAACTGTGGAAG
 121  L  R  G  N  H  E  C  A  S  I   N  R  I  Y  G  F  Y  D  E  C   K  R  R  Y  N  I  K  L  W  K

 537 ACTTTCACCGACTGCTTCAACTGCCTGCCC ATTGCAGCCATTGTAGATGAGAAGATCTTC TGCTGCCACGGAGGCCTGTCTCCAGACTTG
 151  T  F  T  D  C  F  N  C  L  P   I  A  A  I  V  D  E  K  I  F   C  C  H  G  G  L  S  P  D  L

 627 CAATCCATGGAGCAGATTAGACGTATTATG CGGCCCACGGACGTGCCTGACCAGGGCCTG TTGTGTGATCTGCTGTGGTCTGACCCTGAC
 181  Q  S  M  E  Q  I  R  R  I  M   R  P  T  D  V  P  D  Q  G  L   L  C  D  L  L  W  S  D  P  D

 717 AAGGATGTTCAAGGCTGGGGCGAGAATGAC CGTGGAGTCTCCTTTACCTTTGGGGCCGAG GTTGTAGCCAAGTTCCTGCACAAGCATGAT
 211  K  D  V  Q  G  W  G  E  N  D   R  G  V  S  F  T  F  G  A  E   V  V  A  K  F  L  H  K  H  D

 807 TTGGACCTCATCTGCAGAGCACATCAGGTT GTAGAAGATGGCTATGAGTTCTTTGCCAAG AGGCAGCTGGTGACACTCTTCTCAGCCCCC
 241  L  D  L  I  C  R  A  H  Q  V   V  E  D  G  Y  E  F  F  A  K   R  Q  L  V  T  L  F  S  A  P

 897 AACTACTGTGGCGAGTTTGACAACGCTGGC GCCATGATGAGTGTGGACGAGACACTCATG TGTTCCTTCCAGATCCTCAAGCCCGCTGAT
 271  N  Y  C  G  E  F  D  N  A  G   A  M  M  S  V  D  E  T  L  M   C  S  F  Q  I  L  K  P  A  D

 987 AAGAATAAGGGGAAGTATGGGCAGTTCAGT GGCCTGAACCCCGGAGGCCGTCCCATCACT CCACCCCGCAATTCTGCCAAAGCCAAGAAA
 301  K  N  K  G  K  Y  G  Q  F  S   G  L  N  P  G  G  R  P  I  T   P  P  R  N  S  A  K  A  K  K

1077 TAG CCTCCATGTGCTGCCCTCTGCCCCAGATGACGGATTATTTGTACAGAAATCATGCTGCCATGGGTCACACTGGCCTCTCAGGCCCACC
      *

1168 CATCATGGGGAACACAGCGTTAAGTGTCTTTCCTTTATTTTTTAAAGAATCAATAGCAGCATCTAATTCCCCAGGGCTCCCTCCCACCAGCA

1260 CCTGTGGTGGCTGCAAGTGGAATCCTGGGGCCAAGGCTGCAGCTCAGGGCAATGGCAGACCAGATTGTGGGTCTCCAGCCTTGCATGGCTGG

1352 CAGCCAGATCCTGGGGCAACCCATCTGGTCTCTTGAATAAAGGTCAAAGCTGG
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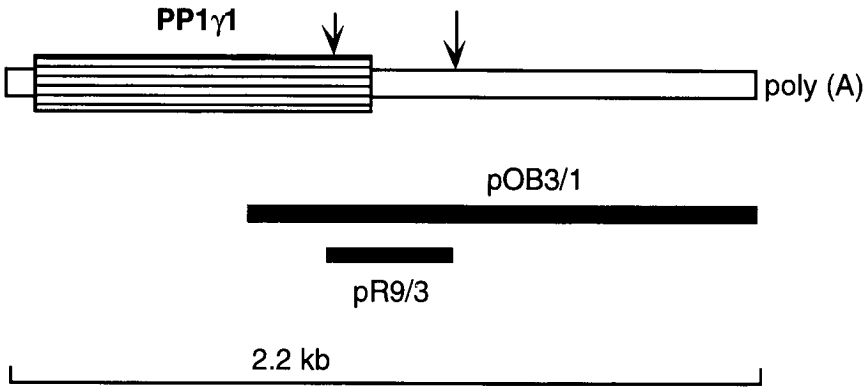
Figure 1. *A*, Diagrammatic representation of the mRNA encoding rat PP1α. The coding region is identified with *horizontal bars*. Partial cDNAs used to derive the cDNA sequence are aligned below. The *arrows* indicate the fragment used to generate riboprobes for *in situ* hybridization. *B*, Nucleotide and deduced amino acid sequence of rat striatum PP1α.

(Piscataway, NJ). Bovine serum albumin (Pentax fraction V), glutaraldehyde, Nonidet P40, polyvinylpyrrolidone, and thyroglobulin were obtained from Sigma (St. Louis, MO). pBluescript and NucTrap columns were purchased from Stratagene (La Jolla, CA).

Cloning of neuronal isoforms of rat PP1. Screening of a rat striatum random-primed cDNA library in λ gt10 (Clontech Laboratories Inc., Palo Alto, CA) was performed essentially as described by Woo (1979). Low stringency hybridization was performed using 10^6 cpm/ml of a PP1 probe prepared by random hexanucleotide priming (Feinberg and Vogelstein, 1983) and purified using a NucTrap probe

A)



B)

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 211   K  D  V  L  G  W  G  E  N  D   R  G  V  S  F  T  F  G  A  E   V  V  A  K  F  L  H  K  H  D

 803  TTGGATCTTATATGTAGAGCCCATCAGGTG GTTGAAGATGGATATGAGTTTTTTGCAAAG AGGCAGTTAGTCACTCTGTTTTCTGCACCC
 241   L  D  L  I  C  R  A  H  Q  V   V  E  D  G  Y  E  F  F  A  K   R  Q  L  V  T  L  F  S  A  P

 893  AACTACTGTGGCGAGTTTGACAATGCGGGC GCCATGATGAGTGTGGATGAGACCCTCATG TGCTCCTTCCAGATTTTAAAGCCTGCAGAG
 271   N  Y  C  G  E  F  D  N  A  G   A  M  M  S  V  D  E  T  L  M   C  S  F  Q  I  L  K  P  A  E

 983  AAAAAGAAGCCCAATGCCACGAGACCTGTC ACACCGCCACGGGGTATGATCACAAAGCAA GCAAAGAAATAG ATGTCACTTGACACTGC
 301   K  K  K  P  N  A  T  R  P  V   T  P  P  R  G  M  I  T  K  Q   A  K  K  *

1072  CTAGTGGGACTTGTAACATAGCGTACATAACCTTCCTTTTTAAACTGTATGTGTGCTGGTCAGCTTGCCCAGGTAGACCTGTCGGGCCCTCC

1164  CTTCTCCATTTTGATTATTGCTGGCACTTGCTGGTTATAGCAGCAAGTGAAGCATTTCATTCTCAAGAAAGCGTTTGTTTTTGTTTGTTTTT

1256  AACCTCTGTTCCCTTTGTGGACAGCTCTGATGATGGTGTTAAGCTGTACACCTTGGCAGTTTATCCTGTCCCAAGTGAAGCATTTCATTCTC

1348  AAGAAAGCGTTTGTTTTTGTTTGTTTTTAACCTCTGTTCCCTTTGTGGACAGCTCGGATATAAAGAGAGCCCTAGGGTGCGTGAGTCTGTAC

1440  ATGTAATTGTCATAAATGCATTCTGTTGATACAAACCACTGTGAACAGTTTTTCCAAGTTTGTTTGACAGGGACTGCTTTCCCTCACTGTCT

1532  CATCCTGTACAAACTAGTGTCTGCAGCTGTGGCAGCAGGAGCAACCTGCCACCCTGCCACCCACACTGCCCAGGCTGTCTGAAGCACACTCC

1624  CTCCCACTGCACACTTAACTGACGATTAAAGCCATTCTTTTCAATGTCTGTGATTCCTTCCTAAAGCCAAAGTTTCTGTTGGACTGTATTGG

1716  CACACCCTGGACATGAGGTGGCCAGGGCACCAAGGCTGCTGGCACAGGCCGCCTCCGTGGGGACTCAGAAAGAAGCAGGTTATTTTAACTAG

1808  CAATAGTGATGTAGTGCTGGGTAAGCTATTAATGGTGGAAGTTAATGAGACATTGTACAGTGCCCATATAGTCTATTCACTGAGTAATCTTT

1900  TTACAGTTGGATCAGGCCTGAACCCGTCCATTCAGAAAGCTTCAAATTATAGAAACAACACTGTCCTATACGAGTGATCGATAATGCTTTCT

1992  TTGGCTACATTCTTTATTCTGCGGTGACATTGAGGCTTATAAATCAAAAGGAACTAACTTGCCGTCCACCGGTTTATACAGAACTCACAGTA

2084  TCTATGACTTTTTTAAACTACGACCTGTTAAAATGAATCTGTTTCCACAGATGCCGTGTACAATGCCATGTGCTAAGAATGATTTCAGACTT

2176  ATTAAATGCGAGCTTGTTAACCTGTCAAAAAAAAAAAAAAAAAA
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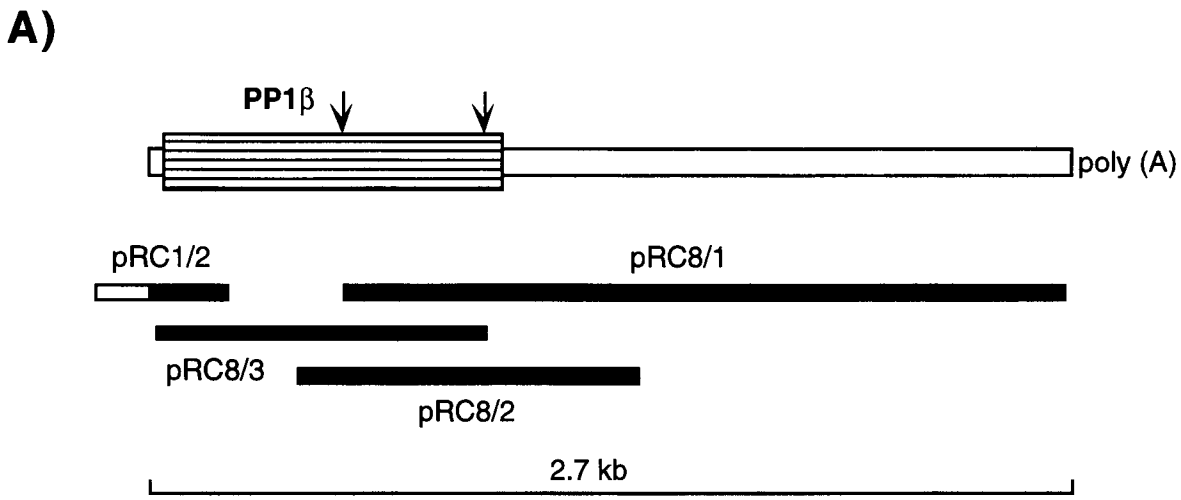
Figure 2. *A*, Diagrammatic representation of the mRNA encoding rat PP1γ1. The coding region is identified with *horizontal bars*. Partial cDNAs used to derive the cDNA sequence are aligned below. The *arrows* indicate the fragment used to generate riboprobes for *in situ* hybridization. *B*, Partial nucleotide and deduced amino acid sequence of rat striatum PP1γ1.

purification column (Stratagene, La Jolla, CA). The probe used consisted of the full length rabbit PP1α cDNA (Bai et al., 1988). Positive clones were purified by successive replating. Single well-isolated positive plaques were picked into 100 μl of λ diluent [10 mM Tris-HCl (pH 7.5)/10 mM $MgSO_4$] and the phage allowed to diffuse out overnight at 4°C. The cDNA inserts were recovered by DNA amplification using 5 μl of phage solution in a 100 μl reaction mixture. The oligonucleotide primers used flank the EcoRI cloning site of the λ gt10 vector (FP10 = 5′-TTG AGC AAG TTC AGC CTG GTT AAG-3′ and RP10 = 5′-GCT TAT GAG TAT TTC TTC CAG GGT-3′). Reaction conditions consisted of 5 min at 94°C followed by 30 cycles of 1 min denaturation at 94°C, 2 min annealing at 65°C and 3 min extension at 72°C, and a final incubation at 72°C for 10 min. Insert sizes were verified by agarose gel electrophoresis of the polymerase chain reaction products. The amplified inserts were either subcloned directly into the EcoRV site of pBluescript-KS^+ or,



B)

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   1                                                                                                    GCTGCGGGGAGGAGAGTCTGGTGCCTACAAG

  32  ATGGCGGACGGGGAGCTGAACGTGGACAGC CTCATCACCCGCCTGCTGGAGGTACGAGGA TGTCGTCCGGGAAAAATTGTGCAGATGACT
   1   M  A  D  G  E  L  N  V  D  S   L  I  T  R  L  L  E  V  R  G   C  R  P  G  K  I  V  Q  M  T

 122  GAAGCAGAAGTCCGAGGACTGTGTATCAAG TCTCGTGAAATCTTTCTTAGCCAGCCTATT CTTTTGGAATTGGAAGCGCCACTGAAGATT
  31   E  A  E  V  R  G  L  C  I  K   S  R  E  I  F  L  S  Q  P  I   L  L  E  L  E  A  P  L  K  I

 212  TGTGGAGATATTCATGGACAGTATACAGAC TTACTGAGATTATTTGAATATGGAGGTTTT CCACCAGAAGCCAACTATCTTTTCTTAGGA
  61   C  G  D  I  H  G  Q  Y  T  D   L  L  R  L  F  E  Y  G  G  F   P  P  E  A  N  Y  L  F  L  G

 302  GATTATGTGGACAGAGGAAAGCAGTCTTTG GAAACCATCTGTTTGCTATTGGCTTACAAA ATCAAATACCCAGAGAACTTCTTTCTTCTA
  91   D  Y  V  D  R  G  K  Q  S  L   E  T  I  C  L  L  L  A  Y  K   I  K  Y  P  E  N  F  F  L  L

 392  CGAGGAAACCATGAGTGTGCTAGCATCAAC CGCATTTATGGATTCTATGATGAGTGCAAA CGAAGATTTAATATTAAATTGTGGAAGACA
 121   R  G  N  H  E  C  A  S  I  N   R  I  Y  G  F  Y  D  E  C  K   R  R  F  N  I  K  L  W  K  T

 482  TTCACTGATTGTTTTAATTGTCTGCCTATA GCTGCTATTGTTGATGAGAAAATCTTCTGC TGTCATGGAGGACTGTCACCAGACCTACAG
 151   F  T  D  C  F  N  C  L  P  I   A  A  I  V  D  E  K  I  F  C   C  H  G  G  L  S  P  D  L  Q

 572  TCTATGGAACAGATTCGGAGAATTATGAGA CCCACTGACGTACCTGATACAGGTTTGCTT TGTGATTTACTGTGGTCCGACCCAGATAAG
 181   S  M  E  Q  I  R  R  I  M  R   P  T  D  V  P  D  T  G  L  L   C  D  L  L  W  S  D  P  D  K

 662  GATGTACAAGGCTGGGGAGAAAATGATCGT GGTGTTTCTTTTACTTTTGGAGCTGATGTA GTCAGTAAATTTCTGAATCGTCATGATTTG
 211   D  V  Q  G  W  G  E  N  D  R   G  V  S  F  T  F  G  A  D  V   V  S  K  F  L  N  R  H  D  L

 752  GACTTGATTTGTCGAGCCCATCAGGTGGTA GAAGATGGATATGAATTTTTTGCTAAACGA CAATTGGTAACTTTATTTTCTGCCCCAAAT
 241   D  L  I  C  R  A  H  Q  V  V   E  D  G  Y  E  F  F  A  K  R   Q  L  V  T  L  F  S  A  P  N

 842  TACTGCGGCGAGTTTGACAATGCTGGTGGT ATGATGAGTGTGGATGAAACTTTGATGTGT TCATTCCAGATATTGAAACCATCTGAAAAG
 271   Y  C  G  E  F  D  N  A  G  G   M  M  S  V  D  E  T  L  M  C   S  F  Q  I  L  K  P  S  E  K

 932  AAAGCTAAGTACCAGTATGGTGGGCTGAAT TCTGGACGTCCTGTCACTCCGCCTCGAACA GCTAATCCACCGAAGAAAAGGTGA AGACA
 301   K  A  K  Y  Q  Y  G  G  L  N   S  G  R  P  V  T  P  P  R  T   A  N  P  P  K  K  R  *
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Figure 3. *A*, Diagrammatic representation of the mRNA encoding rat PP1β. The coding region is identified with *horizontal bars*. Partial cDNAs used to derive the cDNA sequence are aligned below (pRC1/2 was not sequenced in its entirety as indicated by the *open box*). The *arrows* indicate the fragment used to generate riboprobes for *in situ* hybridization. *B*, Nucleotide and deduced amino acid sequence of rat cortex PP1β.

otherwise, they were first digested with EcoRI and then ligated with EcoRI-digested pBluescript-KS$^+$.

An Okayama-Berg rat brain cortex cDNA library was screened for PP1γ using a partial cDNA for PP1γ1 obtained from the rat striatum library described above (pR9/3 in Fig. 2). For sequencing and further manipulations, the positive cDNAs were excised from the pCD vector using the restriction enzyme BamHI and ligated into the same site of pBluescript-KS$^+$.

In order to obtain PP1β cDNA clones, a rat brain cortex cDNA library in λ ZAP II was obtained from Stratagene (La Jolla, CA). Screening was performed in duplicate using PP1β-specific oligonucleotides (Sasaki et al., 1990) directed to either 5′ or 3′ untranslated region sequences (BETA-N = 5′-ATC TTG TAG GCA CCA GAC TCT CCT CCC CGC AGC-3′ and BETA-C = 5′-AGG GTG TCG AAT GGC TGG ATG GTT TTA CAG TGC-3′). Oligonucleotides were labeled as follows: 40 pmol of either oligonucleotide was incubated for 30 min at 37°C in 50 μl of 60 mM Tris-HCl (pH 7.8)/10 mM $MgCl_2$/14 mM β-mercaptoethanol containing 120 μCi of γ-^{32}P-ATP and 20 U of T4 polynucleotide kinase. These conditions resulted in virtually stoichiometric incorporation of label into the oligonucleotides, as determined by chromatography on Sephadex G-25 and polyacrylamide gel electrophoresis, and the probes were used without further purification. Prehybridization

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1021  GGAATTCCGGAAAGAGAAACCATCAGATTTGTTAAGGACATACTTCATAATATATAAGTGTGCACTGTAAAACCATCCAGCCATTCGACACC

1113  CTTTATGATGTCACACCTTTAACTTAAGGAGACGGGTAAAGGATCTTAAATTTTTTTCTAATAGAAAGATGTGCTACACTGTATTGTAATAA

1205  GTATACTCTGTTATAATATTCAACAAAGTTAAATCCAAATTCAAAAGTATCCATTAAAGTTCTATCTTCTCATATCACAGTTTTTAAAGTTG

1297  AAAGCATCCCAGTTAAACTAGCTGCGTTAGTTACCCAGATGAGAGCATGAAGATCCATCTGTGTAATGTGGCTTTAGTGTTGCTTGGTTGTT

1389  TCTTTATTTTGGGCTTGTTTTGTTTTGTTTGTTTTTGCTAGAATAATGGCATCTACTTTTCCTATTTTTCCCTAACCATTTTAAAAAGTGAA

1481  AATGGGAAGAGCTTTAAAGACATTCACCAACTATTCTTTTCCTTCACTTATCTACTTAAGGAACTGTTGGATCTTACTAAGAAAACTTACGC

1573  CTCATAATAAAAAGGAACTTTAGAGGCCGATAGGTTTTAAAAATATACAAACTATTTGATCCAATGATTTTAATCAAACAGTTTGACTGGGC

1665  AAACTTTGCAGCTGATAATGACTATTTCGCTTTTTACAAATTGCCACTGATTTGGATTTGTGCACTCTAACCTTTAATTTATTGATGCTCTA

1757  TTGTGCAGTAGCATTTCATTTAAGATAAGGCTCATATAGTACTATCCAAAATTAGTTGGTAATGTGATTATGTGGTACCTTGGCTTTAGGTT

1849  TTAATTCGCACGAAACACCTTTTGGCATGCTTAACTTTCTGGTATTATCCTCACCTGCATTGGTTTTGTTTTTTGGGGTTTTTGTTGTTGTT

1941  TGTTTGTTTGTTTTTAGATCCACAGAACATGAGAATCCTTTTTGACAAGCCTTGGAAAGCTGGCTCTTCTTTCCCTCTCTATGTGAAGGATG

2033  TATTTAAATGAACACTGGTCAGTGGGACATTGTCAGCTCTGAGTATTGGGTGCTTCACTGTCTAATAATTGCCATGTGAATGTTGTTTTTGA

2125  CTGTAAGGCTATGTCACTAAAGATTTTTACTCTGCGTTTTCATAATCAAAGGTCATGATGTGTATAGACATGCTTTGTAGTGAAGTATAGTA

2217  GCAATAATTTCTGCACATGATCAAGAGTTTATTGCAGCATTTCTTTCCCTGTTCTCTCTTTTTTAAGGGTTAGCATTAACAAATGTCAAGGA

2309  ATAGCAAAGTCAACAAAGACTTTAGGAGGTGGAATTAAGAACACACAGATTTGTGATCTTTGGATGTGACACTTATTGGATGTTATTCTAAA

2401  GTCTTATTGAACATTGTCAAATTTGTAAGCTTCATGGGGATGGACATAATGTTTATATAATGCCCTTCTTATGTGTTACCATAGATGTGAAA

2493  CCTTATATTGTCTTTGAAAATGTTAAATTGAGAACTCTGTTAACATTTTATGGATTGGCACATTATATTACTGCAAGAAACATTTGATTTTG

2585  AGCACAGTGCAAAAGTTCTTTAAAATGCATATGTCTTTTTTTCTAATTCAATTTTGTTTAAAGCACATTTTAAATGTAGTTTTC
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Figure 3. Continued.

and hybridization were performed at 42°C in a buffer consisting of 90 mM Tris-HCl (pH 7.4)/0.9 M NaCl/6 mM EDTA/0.5% (v/v) Nonidet P40/2× Denhardt's solution/0.2% (w/v) SDS/0.05% (w/v) sodium pyrophosphate/70 μg/ml *E. coli* tRNA/100 μg/ml denatured salmon sperm DNA. Hybridization was carried out with 0.8 pmol ^{32}P-labeled oligonucleotide/ml for 12–20 hr. Positive clones were identified, purified by successive replating and their cDNA inserts recovered by *in vivo* excision into pBluescript-SK$^-$.

DNA sequencing. Double stranded plasmid DNA purified on Magic Miniprep columns (Promega Corporation, Madison, WI) was sequenced in the presence of 7-deaza-2′-dGTP (Sanger et al., 1977; Mizusawa et al., 1986). Plasmid DNA (1–2 μg) was denatured in 0.2 M NaOH/0.2 mM EDTA for 5 min at room temperature, neutralized with 0.1 vol of 2 M ammonium acetate (pH 4.8) and precipitated with 2.5 vol of 100% ethanol. DNA was recovered by centrifugation, rinsed with 75% ethanol, dried under vacuum, and used for sequencing with Sequenase (United States Biochemicals, Cleveland, OH) under the manufacturer's recommended conditions. The sequencing primers used consisted of either pBluescript-specific or cDNA-specific oligonucleotides.

In situ *hybridization.* Adult male Sprague–Dawley rats (Charles River Laboratories, 250 gm) were sacrificed by decapitation. Their brains were rapidly removed, frozen in liquid isopentane (−30°C) for 30 sec and stored at −80°C until sectioning. Frozen tissue blocks were warmed to −20°C and mounted on a chuck with M-1 mounting medium (Lipshaw). Serial coronal sections (10 μm) were prepared using a Bright cryostat, thaw mounted (two sections per slide) onto polylysine subbed microscope slides and stored at −80°C.

Adjacent sections were processed for PP1α, PP1β, or PP1γ1 *in situ* hybridization. Sections were removed from storage, fixed in 4% (w/v) formaldehyde for 60 min at room temperature and then rinsed three times in 2× SSC [300 mM NaCl/30 mM Na citrate (pH 7.2)]. Sections were treated with proteinase K [1 μg/ml in 100 mM Tris-HCl (pH 8.0)/50 mM EDTA] for 10 min at 37°C, rinsed in water and treated with a mixture of 0.1 M triethanolamine (pH 8.0) and acetic anhydride (400: 1, v/v) with stirring for 10 min. The sections were rinsed in 2× SSC, dehydrated through graded alcohols, and allowed to air dry.

Serial brain sections were hybridized with ^{35}S-UTP-labeled riboprobes specific for each of the isoforms of protein phosphatase 1. For this purpose, the following cDNA fragments were subcloned into pBluescript: (1) PP1α, a 478 nt PstI fragment from pR14/1 (Fig. 1*A*); (2) PP1γ1, a 377 nt EcoRI fragment from pR9/3 (Fig. 2*A*); (3) PP1β, a 423 nt EcoRI fragment from pRC8/1 (Fig. 3*A*).

Sense and antisense cRNA probes were generated by *in vitro* transcription of the linearized constructs, diluted in hybridization buffer [75% (v/v) formamide/10% (w/v) dextran sulfate/3× SSC/50 mM Na_2PO_4 (pH 7.4)/1× Denhardt's/0.1 mg/ml yeast tRNA/10 mM dithiothreitol] and applied to the sections to a final concentration of 1–2 × 10^6 dpm/30 μl. Thirty microliters of the hybridization solution were used per slide (containing two coronal sections), a coverslip was placed on top and the sections were hybridized overnight at 55°C in a humid chamber.

After hybridization, the sections were rinsed three times in 2× SSC, treated with RNase A [200 μg/ml in 100 mM Tris-HCl (pH 8.0)/0.5 M NaCl] for 1 hr at 37°C, and rinsed again in 2× SSC. The sections were then washed at room temperature in 1× SSC for 5 min, 0.5× SSC for 5 min, and 0.1× SSC for 5 min. Finally, the sections were washed in 0.1× SSC at 65°C for 1 hr, rinsed in water at room temperature, dehydrated through graded alcohols, and air dried. The sections were then exposed to Kodak XAR-5 x-ray film for 8 d. Afterwards the sections were counterstained with cresyl violet.

The specificity of the *in situ* hybridization signal was verified using two sets of controls for each protein phosphatase clone. One set was used for RNase controls as follows. The sections were treated prior to hybridization with RNase A (200 μg/ml) for 1 hr at 37°C, before the proteinase K treatment. The control sections were then processed as described above and hybridized with either the PP1α, PP1β, or PP1γ1 cRNA probes. No specific signal was observed on RNase A pretreated sections. The other set was processed for sense probe controls. Tissue sections were processed for *in situ* hybridization as described above. However, sense cRNA probes with sequences identical to the PP1 mRNA sequences targeted by the antisense riboprobes were used. No specific signal was observed on sections hybridized with these sense strand probes.

Synthesis of peptides. Synthetic peptides corresponding to C-terminal

sequences for the α and the γ1 isoforms were synthesized at the Keck Foundation Biopolymer Facility (Yale University), and the PP1β peptide was synthesized at the Rockefeller University Protein Sequencing Facility. All peptides, purified by reverse-phase HPLC to >95% purity, yielded the expected amino acid composition and mass spectra (data not shown).

Conjugation of peptides and production of antibodies. The purified peptides were conjugated to bovine thyroglobulin at a peptide:carrier ratio of 25:1. Thyroglobulin (33 mg) was dissolved in 2 ml of 0.125 M sodium phosphate buffer (pH 7.5) and filtered through a 0.22 μm Millex GV syringe filter. Peptide (1.25 μmol) dissolved in water was added directly to the thyroglobulin and the solution was allowed to cool to 4°C. Conjugation was initiated with the dropwise addition, over 20 min, of 2.5 ml of freshly diluted glutaraldehyde, and the reaction was allowed to proceed for a total of 2 hr at 4°C with constant stirring. The reaction was quenched with 150 μl of sodium borohydride (12.5 mg/ml) and stirred for a further 30 min at 4°C. Finally, the solution was dialyzed overnight against PBS (137 mM NaCl/5.4 mM Na_2HPO_4/2.7 mM KCl/1.8 mM KH_2PO_4, pH 7.4), aliquoted, and stored at −70°C until needed for immunization.

Antisera against PP1 isoforms were obtained by injecting New Zealand White female rabbits intradermally at multiple sites with the conjugated peptides mixed with complete Freund's adjuvant. Initially, three rabbits were injected with each conjugated peptide. Each rabbit was then boosted on day 14 and day 21 with conjugated peptide mixed with incomplete Freunds adjuvant. Test bleeds were performed on day 28. The titer and specificity of this and subsequent weekly bleeds was assessed by immunoblotting against striatal homogenates and peptide dot blots. The sera showing the highest titer and specificity were chosen for affinity purification and further characterization.

Peptide affinity columns were prepared using the same peptides that were used to raise antibodies. The peptides were coupled to activated CH-Sepharose 4B under the manufacturer's recommended conditions and packed into a 5 ml column. The serum was filtered through 0.22 μm Millex-GV filters and protease inhibitors were added to the following final concentrations: 25 mM benzamidine, 5 mM EDTA, 1 mM EGTA, 100 μM PMSF, 20 μg/ml leupeptin, 20 μg/ml antipain, 5 μg/ml pepstatin, 5 μg/ml chymostatin. Each sample was then applied to its respective affinity column and the flowthrough was reapplied to the column. After washing to ensure the removal of nonspecific IgG, specific antibodies were eluted with 4.3 M $MgCl_2$ and dialyzed against PBS overnight. Antibody solutions were concentrated by Centriprep (Amicon, Beverly, MA) centrifugation and their concentration was calculated from the absorbance at 280 nm, assuming a 1 mg/ml IgG solution to have an absorbance of 1.4. The specificity of the affinity purified antibodies was checked by immunoblotting against recombinant PP1 isoforms produced in insect Sf9 cells infected with the appropriate baculovirus (E. F. da Cruz e Silva and P. Greengard, unpublished observations). Aliquots were stored at −70°C.

Immunoblotting. Young adult male Sprague–Dawley rats were sacrificed by CO_2 asphyxiation and various brain and peripheral tissues were rapidly dissected on ice. Tissue samples were frozen immediately on dry ice. Boiling 1% (w/v) SDS was added and the samples homogenized by sonication. Insoluble material was removed by centrifugation at 12,000 × *g* for 5 min, and the protein concentration of the supernatants measured by the bicinchoninic acid method (Pierce, Rockford, IL) as described by Smith et al. (1985). Aliquots were separated on 12% SDS-PAGE gels and transferred electrophoretically onto 0.2 μm pore size nitrocellulose membranes (Schleicher and Schuell, Keene, NH) at 200 mA for 12–16 hr in 20 mM Tris-HCl (pH 8.6)/150 mM glycine, containing 20% (v/v) methanol (Towbin et al., 1979). Following transfer, nonspecific binding sites on the membranes were quenched at room temperature for 1 hr in TBS [50 mM Tris-HCl (pH 7.6)/150 mM NaCl/0.05% (w/v) sodium azide)/Tween [TBS/Tween = TBS + 0.05% (v/v) Tween 20] containing 2% (w/v) Carnation dried milk. Membranes were incubated in the same solution containing antiserum at the appropriate dilution for 1–2 hr at room temperature. The membranes were washed 3 × 10 min with TBS/Tween and the bound antibodies were detected by radiolabeling with ^{125}I-protein A, diluted 1000-fold in TBS/Tween + 2% (w/v) milk. Finally, the blots were washed at room temperature 4 × 10 min with TBS/Tween, 2 × 5 min with TBS, dried and exposed at −70°C to Kodak X-Omat AR film using Du Pont Lighting Plus intensifying screens. For quantitative purposes, a PhosphoImager was used (Molecular Dynamics, Sunnyvale, CA).

Kainic acid lesion experiments. Male Sprague–Dawley rats (150–200 gm) were anesthetized with 400 mg/kg (i.p.) chloral hydrate, and kainic acid lesions were performed as previously described (Walaas and Greengard, 1984). The right caudate-putamen (neostriatum) was infused with 1 μl of kainic acid (1 μg/μl in PBS) at a rate of 0.5 μl/min, using stereotactic coordinates derived from König and Klippel (1963).

The animals were sacrificed 8–11 d postsurgery, and the striatum and substantia nigra were dissected from both sides of the brain and frozen immediately on dry ice. Homogenates were prepared immediately by sonication in boiling 1% (w/v) SDS and protein quantitation was performed by the bicinchoninic acid method (Smith et al., 1985). Samples were then analyzed by SDS-PAGE followed by immunolabeling with the PP1 isoform-specific antibodies as described above. As controls, duplicate samples were analyzed using anti-DARPP-32, anti-GFAP, anti-actin, and anti-tubulin antibodies.

Results

Cloning of neuronal isoforms of rat PP1

Low stringency screening of a rat λ gt10 striatal cDNA library yielded a large number of positive clones. Sequence analysis revealed that they all encoded partial sequences derived either from PP1α or PP1γ1 (see Discussion). Figure 1 shows the deduced full length sequence of rat PP1α derived from a number of overlapping clones. In this initial screen a cDNA clone partially encoding PP1γ1 was isolated (pR9/3) and used to screen a rat cortex Okayama-Berg cDNA library, thought to be more likely to contain full length clones. Although two different clones were isolated from this library, sequence analysis indicated that they too were not full length. Figure 2 shows the partial sequence obtained for rat PP1γ1 from the various overlapping cDNAs. Since no PP1β clones were identified in either of the previous libraries, a rat cortex cDNA library in λ ZAP II was subsequently screened using oligonucleotides whose sequences were derived from the rat liver PP1β cDNA sequence published by Sasaki et al. (1990). The full length sequence of rat cortex PP1β derived from overlapping clones is shown in Figure 3. The cDNA and deduced amino acid sequences of rat brain PP1α, PP1β, and PP1γ1 were found to be identical to those reported by Sasaki et al. (1990) for rat liver.

In situ *hybridization*

The three PP1 cRNA probes used for *in situ* hybridization were similar in size (377–478 nucleotides) and were labeled to similar levels of specific activity. Therefore, the differences observed in signal intensity and distribution suggest that each PP1 isoform has a relatively unique distribution in the rat brain (Figs. 4–8).

Telencephalon. PP1α, PP1β, and PP1γ1 mRNAs were found in several olfactory structures, including the olfactory bulb, olfactory tubercle (Tu), anterior olfactory nucleus (AO), piriform cortex (PO), and nucleus of the lateral olfactory tract (Figs. 4, 5). Although all three isoforms were found in these regions, PP1γ1 was most abundant followed by PP1β and then PP1α. PP1γ1 seemed to be uniquely expressed in the islands of Calleja (ICj), whereas PP1α and PP1β were largely absent. PP1 mRNA was also abundant in the striatum, with PP1α and PP1γ1 being the two predominant isoforms in the caudate-putamen (CPu) and the nucleus accumbens (Acb, Fig. 5). The globus pallidus, which is a major target of striatal efferents, was virtually devoid of PP1α and PP1γ1 mRNA and contained only a small amount of PP1β mRNA. Overall, the highest levels of PP1 mRNA in rat brain were found in the hippocampus. All three PP1 isoforms were abundant in the four CA subfields as well as the dentate gyrus (DG; Figs. 6, 7). All three isoforms of PP1 were expressed in nuclei of the amygdaloid complex (AMY), but the messenger RNAs encoding PP1β and PP1γ1 were expressed to a higher

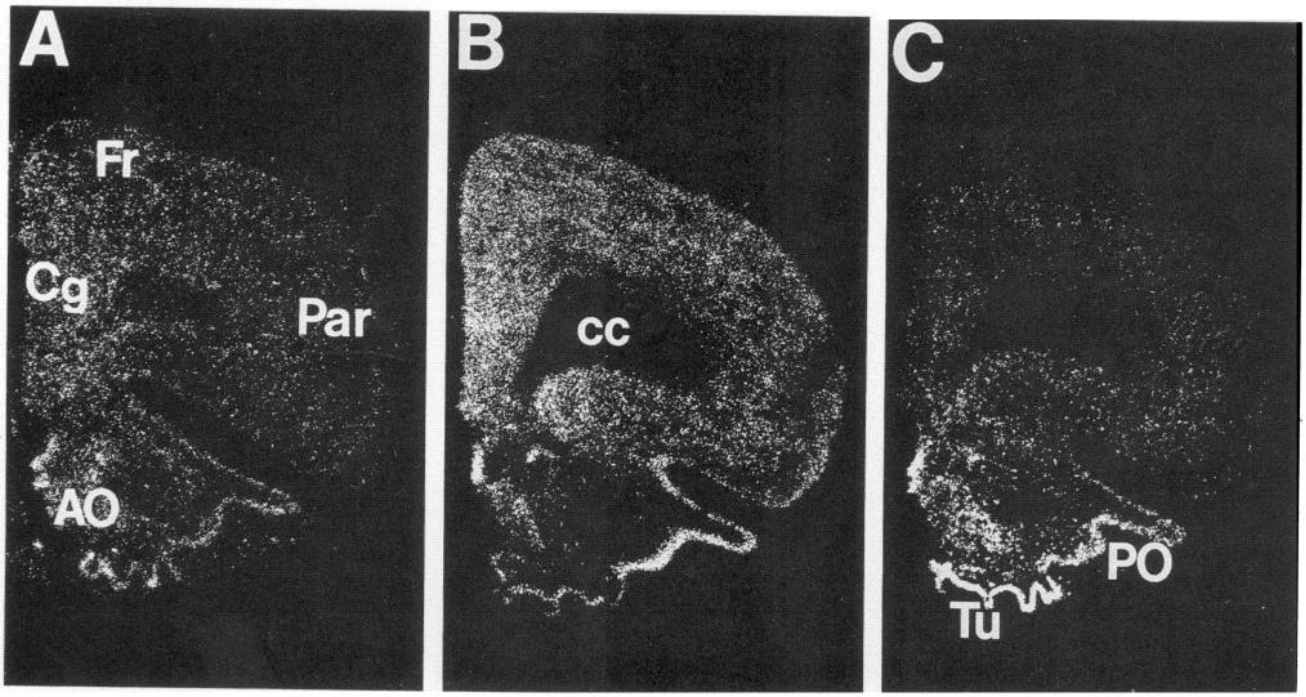

Figure 4. Autoradiographic images of coronal slices through the rostral forebrain showing *in situ* hybridization of PP1α (*A*), PP1β (*B*), and PP1γ1 (*C*) mRNA. PP1α mRNA generally had a lower level of signal at this level, but was moderately expressed in the anterior olfactory nucleus (*AO*), olfactory tubercle (*Tu*), and primary olfactory cortex (*PO*). PP1β mRNA had a high level of expression in the PO, but a lower level of expression in the Tu. *In situ* hybridization for PP1γ1 mRNA produced the highest level of signal, specifically in the Tu, PO, and AO. PP1α, PP1β and PP1γ1 mRNAs were found dispersed throughout layers 2–6 of the cerebral cortex at all levels examined.

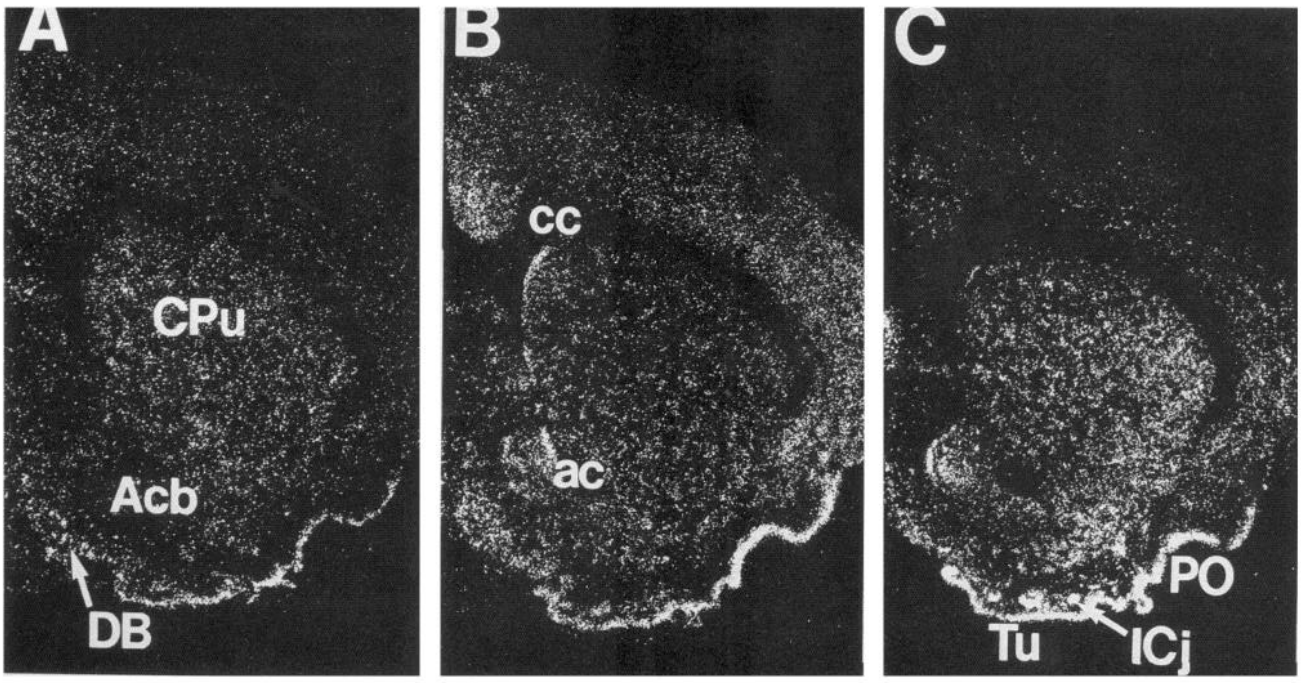

Figure 5. Autoradiographic images of coronal sections through the forebrain at the level of the mid striatum showing *in situ* hybridization of PP1α (*A*), PP1β (*B*), and PP1γ1 (*C*) mRNA. PP1α mRNA was expressed in the caudate-putamen (*CPu*), diagonal band of Broca (*DB*), primary olfactory cortex (*PO*) and olfactory tubercle (*Tu*). PP1β mRNA was expressed distinctly in the DB, PO, and Tu, including low levels in the islands of Calleja (*ICj*), but only low levels were observed in the CPu. PP1γ1 mRNA was found in the CPu, PO, Tu, and ICj.

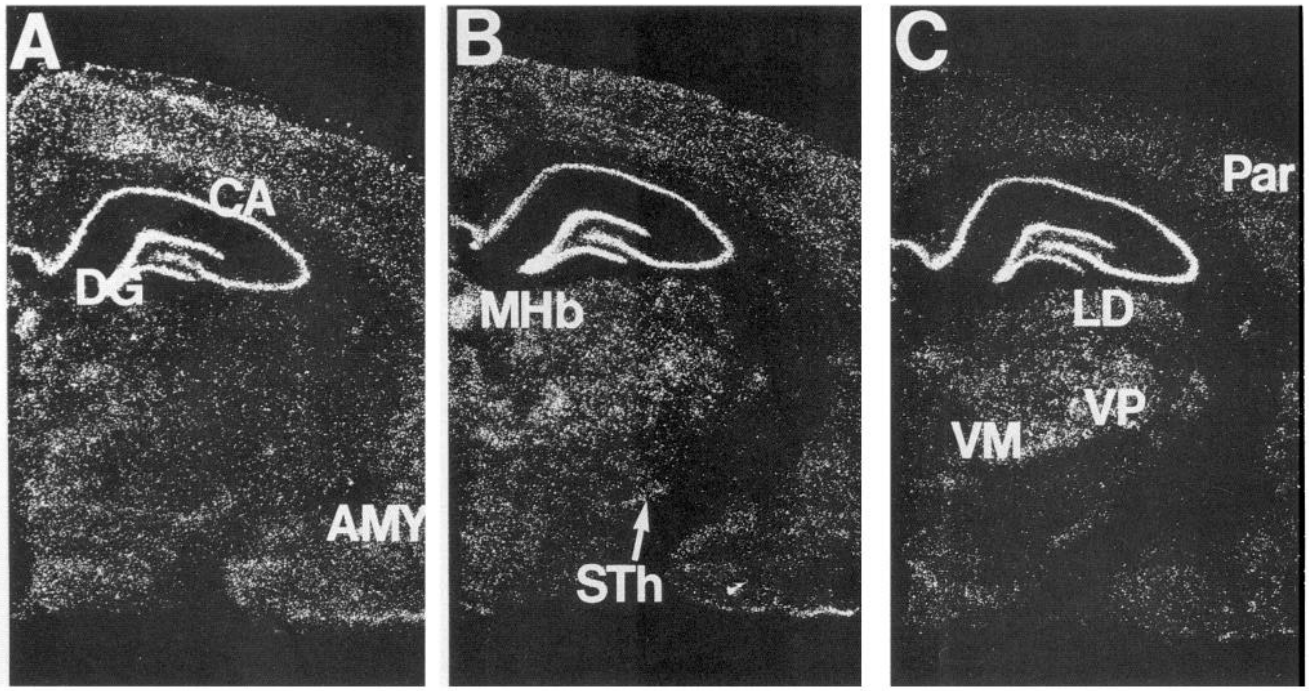

Figure 6. Autoradiographic images of coronal sections through the forebrain at the level of the mid hypothalamus showing *in situ* hybridization of PP1α (*A*), PP1β (*B*), and PP1γ1 (*C*) mRNA. PP1α, PP1β, and PP1γ1 mRNAs were expressed at high levels in all hippocampal subregions (CA fields 1–4 of Ammon's horn, *CA*; dentate gyrus, *DG*). PP1β and PP1γ1 mRNAs were both distributed throughout the thalamus. The amygdaloid complex (*AMY*) contained low to moderate amounts of PP1α, PP1β, and PP1γ1 mRNA. All three isoforms were only expressed at low levels in the hypothalamus.

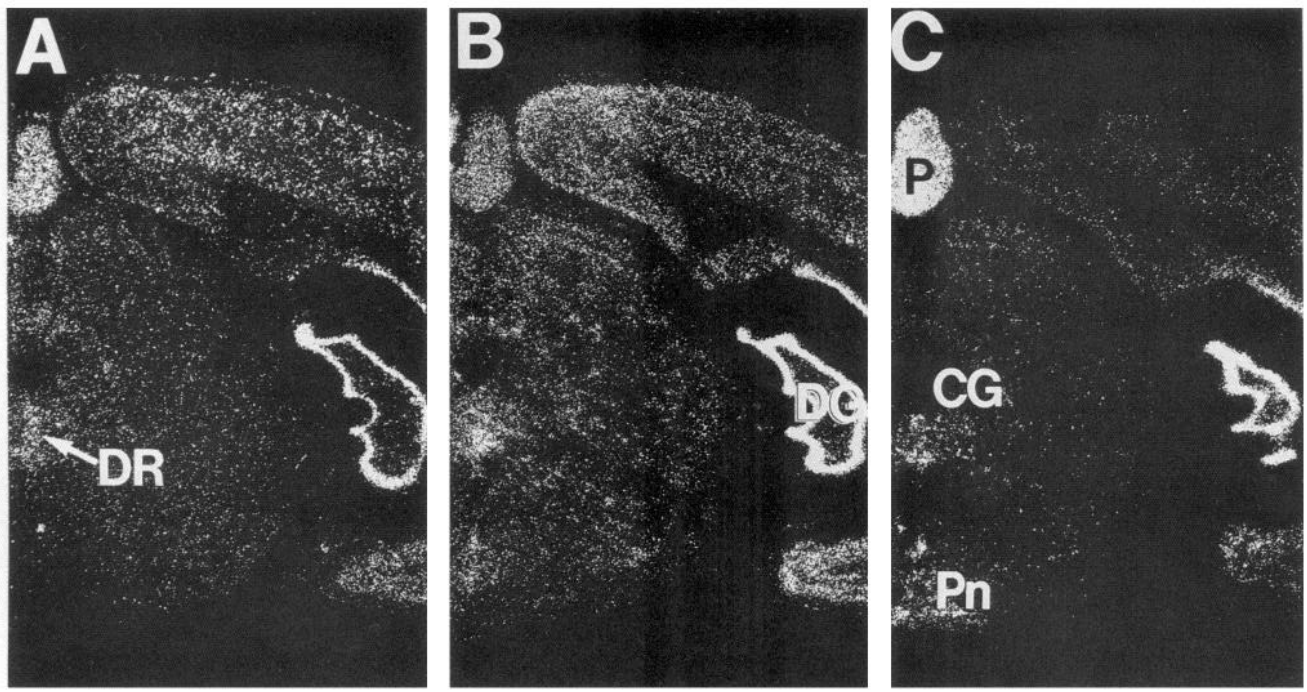

Figure 7. Autoradiographic images of coronal sections through the midbrain at the level of the pons showing *in situ* hybridization of PP1α (*A*), PP1β (*B*), and PP1γ1 (*C*) mRNA. PP1α, PP1β, and PP1γ1 mRNAs were present in the dorsal raphe (*DR*) and hippocampus, which is mostly dentate gyrus in this plane of section. PP1α, and especially PP1γ1 mRNA, showed robust expression in the pineal gland (*P*). All three PP1 isoforms were localized in the central gray (*CG*) and PP1γ1 was also found in the pontine nuclei (*Pn*).

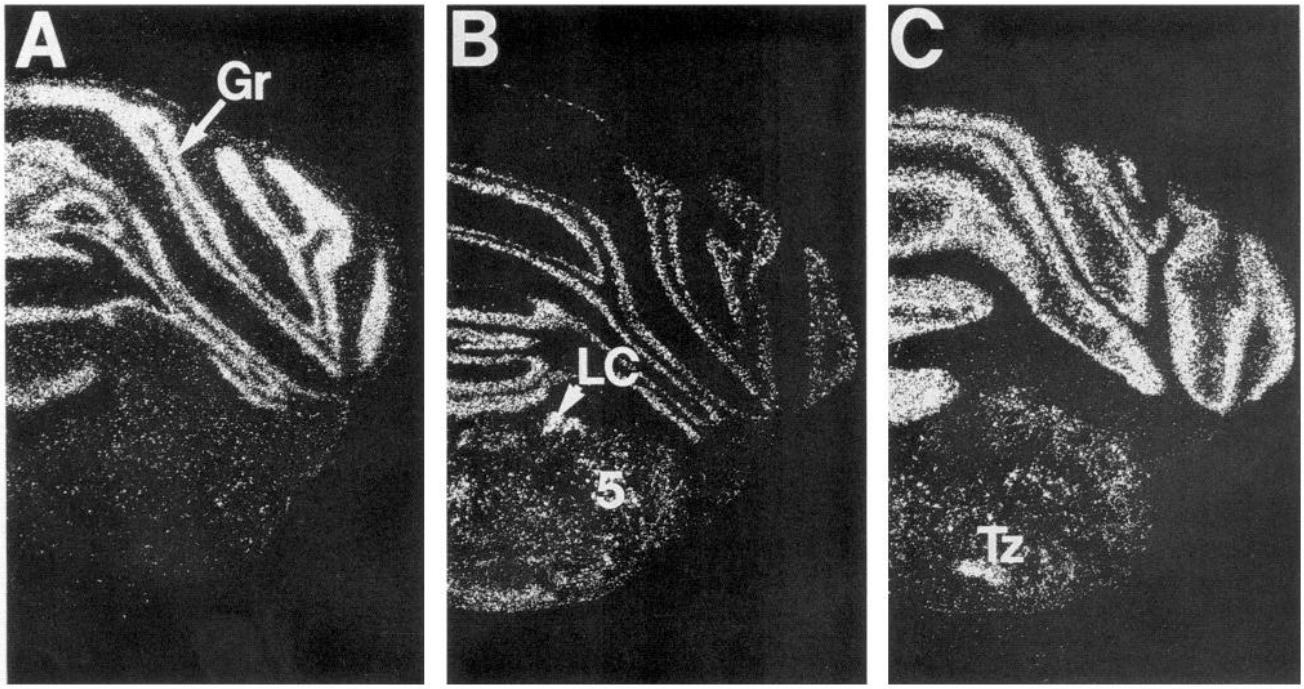

Figure 8. Autoradiographic images of coronal sections through the brainstem and cerebellum showing *in situ* hybridization of PP1α (*A*), PP1β (*B*), and PP1γ1 (*C*) mRNA. All three PP1 isoform mRNAs showed high expression in the granular layer of the cerebellum (*Gr*). PP1β and PP1γ1 mRNAs were both found to have a broad distribution in the brainstem. At this level a high amount of PP1β mRNA was observed in the locus coeruleus (*LC*) and trigeminal nucleus (*5*). Exceptional levels of PP1γ1 mRNA were observed in the nucleus of the trapezoid body (*Tz*) at this level.

degree than PP1α mRNA (Fig. 6). In addition, the amygdalohippocampal transition area expressed high levels of all three PP1 mRNAs. PP1α mRNA was found throughout the bed nucleus of the stria terminalis, PP1γ1 mRNA was expressed to a lesser degree and PP1β mRNA was localized only in the ventral division of this nuclear complex (data not shown). PP1α, PP1β, and PP1γ1 mRNA were detected in layers 2–6 of the cerebral cortex at all levels of the rat brain (Cg, Fr, Par; Figs. 4–7). There was little evidence for PP1 mRNA expression in a specific cortical layer, except in allocortical regions such as the piriform and hippocampal cortex, which are dominated by a single dense cell layer. The septum expressed moderate amounts of PP1α and PP1γ1 mRNA, and the diagonal band of Broca (DB, Fig. 5) contained moderate amounts of all three PP1 mRNAs (vertical and horizontal limbs).

Diencephalon. In general, the thalamus contained relatively high levels of PP1β and PP1γ1 mRNAs, but only low levels of PP1α (Fig. 6). PP1α mRNA was found mainly in medial and midline nuclei. The medial habenula (MHb) was the only region of the thalamus that expressed high levels of PP1α. PP1β mRNA labeling was more widespread in the thalamus, with particularly high levels of expression in the zona incerta and MHb. PP1γ1 mRNA, like PP1β, exhibited a wide thalamic distribution, but it was conspicuously abundant in the medial geniculate body and the MHb (not shown). The subthalamic nucleus (STh) contained all three PP1 mRNAs (Fig. 6). In general, the preoptic area and hypothalamus contained moderate amounts of PP1α and PP1β mRNA, and a low amount of PP1γ1 mRNA (Fig. 6). Hypothalamic nuclei expressing all three PP1 isoforms include the suprachiasmatic nucleus, supraoptic nucleus, paraventricular nucleus, ventromedial nucleus, and the arcuate nucleus.

Mesencephalon. The midbrain dopaminergic nuclei, the substantia nigra and ventral tegmental area, showed a distinctive distribution of PP1 mRNA (not shown). PP1α and PP1β mRNAs were localized in the substantia nigra, pars compacta and lower amounts were detected in the ventral tegmental area. No PP1α or PP1β mRNA expression was observed in the substantia nigra, pars reticulata. PP1γ1 mRNA was also found within the ventral tegmental area, and within both the substantia nigra, pars reticulata and compacta. In addition to the midbrain dopaminergic nuclei, PP1γ1 mRNA was expressed at high levels in the red nucleus and the oculomotor nucleus, and at moderate levels in the interpeduncular nucleus. Both PP1β and PP1γ1 mRNAs were detected in the central gray (CG, Fig. 7). The pineal gland (P) exhibited robust expression of PP1α and particularly PP1γ1 (Fig. 7). However, a relatively low level of PP1β was detected in the pineal gland. PP1γ1 mRNA was also detected in the pontine nuclei (Pn, Fig. 7).

Rhombencephalon. Only a low level of PP1α mRNA was observed in pons and medulla. In contrast, PP1β and PP1γ1 mRNAs were broadly distributed in the pons and medulla. Higher than background levels of expression of PP1β and PP1γ1 mRNAs were detected in several cell groups, including the locus coeruleus (LC, Fig. 8) which is a primary source of noradrenergic neurons in the brain, the trigeminal nuclear complex (5, Fig. 8), and the raphe nuclei, including the dorsal (DR, Fig. 7) and median raphe (data not shown), which are two major sources of serotonergic neurons. PP1α was also localized to the dorsal (Fig. 7) and median raphe. In addition, PP1γ1 mRNA was localized in the vestibular nuclear complex. More caudally in the brainstem, PP1β and PP1γ1 mRNA were localized in the nucleus of the trapezoid body (Tz), the lateral reticular nucleus and

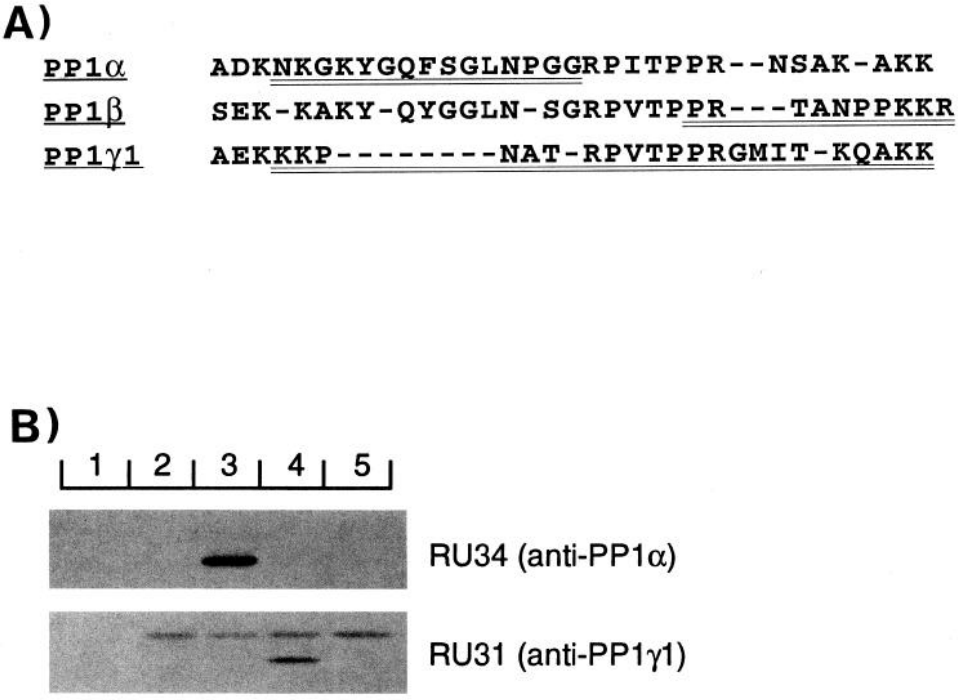

Figure 9. *A*, The deduced C-terminal sequences for neuronal PP1α, PP1β, and PP1γ1 were aligned visually, and the peptides used for antibody production are *double underlined*. Optimal alignment necessitated the introduction of gaps as shown. *B*, Immunoblot analysis demonstrating the specificity of the isoform-specific PP1 antibodies. Gels were loaded with the homogenates of Sf9 insect cells as follows: *1*, uninfected control; *2*, cells infected with wild-type virus; *3*, cells expressing recombinant PP1α; *4*, cells expressing recombinant PP1γ1; *5*, cells expressing recombinant PP1β. See text for description of immunoreactive bands.

the inferior olivary complex. PP1β mRNA was expressed at an exceptional level in a few other brainstem nuclei, including the area postrema and the hypoglossal nucleus (data not shown).

The cerebellum is probably the second richest brain structure in PP1 mRNA, after the hippocampus. All three PP1 mRNAs were found to be very abundant in the granular layer of the cerebellum (Gr, Fig. 8). In addition, the molecular layer of the cerebellum contained low amounts of PP1γ1 mRNA. PP1α and PP1β were not detected in the molecular layer.

Choroid plexus. PP1α, PP1β, and PP1γ1 mRNAs were all expressed in the choroid plexus of the rat brain (data not shown), which is also known to express relatively high levels of both DARPP-32 and inhibitor 1.

Production of isoform-specific antibodies

The availability of the deduced amino acid sequences for the three isoforms of PP1 allowed us to design isoform-specific peptides. Given the high degree of identity between PP1α, PP1β and PP1γ1, their C-terminal regions were chosen since they exhibited the greatest divergence. Figure 9*A* shows the alignment of their deduced C-terminal sequences, and highlights the peptides chosen for antibody production. Preliminary experiments identified RU34 and RU31 as the sera showing the highest titer and specificity against PP1α and PP1γ1, respectively (data not shown). They were then purified by peptide affinity chromatography. The isoform specificity of the affinity purified antibodies is demonstrated in Figure 9*B*. Both RU34 (anti-PP1α) and RU31 (anti-PP1γ1) reacted specifically and strongly against the isoform to which they were raised and showed essentially no cross-reactivity against the other isoform. RU31 (anti-PP1γ) was found to cross-react with a protein present in infected Sf9 cells but not in uninfected cells and, therefore, likely to be encoded in the baculovirus genome. This antibody failed to cross-react with any other mammalian protein (see Fig. 11), raising the possibility of the existence of a baculoviral phosphatase related to PP1.

Whereas it was relatively simple to obtain antibodies specific for PP1α and PP1γ1, we were unable to obtain PP1β-specific antibodies using the peptide described and identical methodology.

Immunoblot analysis of PP1 expression

The availability of isoform-specific antibodies allowed an investigation of the expression of PP1 isoforms in different brain regions and non-neuronal tissues by immunoblotting. Previous studies of neuronal PP1 were mainly concerned with measurement of enzyme activity (Ingebritsen et al., 1983; Nairn et al., 1988), which is not a good indicator of protein abundance and fails to address the existence of different, closely related isoforms. Both antibodies identified a single protein of approximately 37 kDa as predicted from the deduced amino acid sequence (Figs. 10, 11). PP1α and PP1γ1 were found to be expressed in all brain areas analyzed, with the highest levels of expression being observed in the striatum (Figs. 10–12). The isoform distribution was similar in the various regions tested, with three notable exceptions. PP1γ1 was relatively more abundant than PP1α in the olfactory bulb, olfactory tubercle and pineal gland. RU34 (anti-PP1α) and RU31 (anti-PP1γ) also detected 37 kDa proteins in all non-neuronal tissues examined (Figs. 10, 11). In general, PP1α and PP1γ1 abundance was lower in non-neuronal tissues than in brain (Figs. 10–12). Of all non-neuronal tissues analyzed, PP1α and PP1γ1 showed the highest levels of expression in thymus, kidney, spleen, and intestine (Fig. 12). Interestingly, RU34 (anti-PP1α) identified an additional protein of about 30 kDa in rat kidney (Fig. 10). The expression of this protein of aberrant molecular mass is restricted to renal cortex, being largely absent from the medulla (D. Li, G. Celsi, E. F. da Cruz e Silva, P. Greengard, A. Aperia, and B. Meister, unpublished observations). RU31 (anti-PP1γ) identified a protein of about 40 kDa present only in testis of all tissues examined (Fig. 11), which likely represents the alternatively spliced, testis-specific PP1γ2 isoform. Table 1 compares the ex-

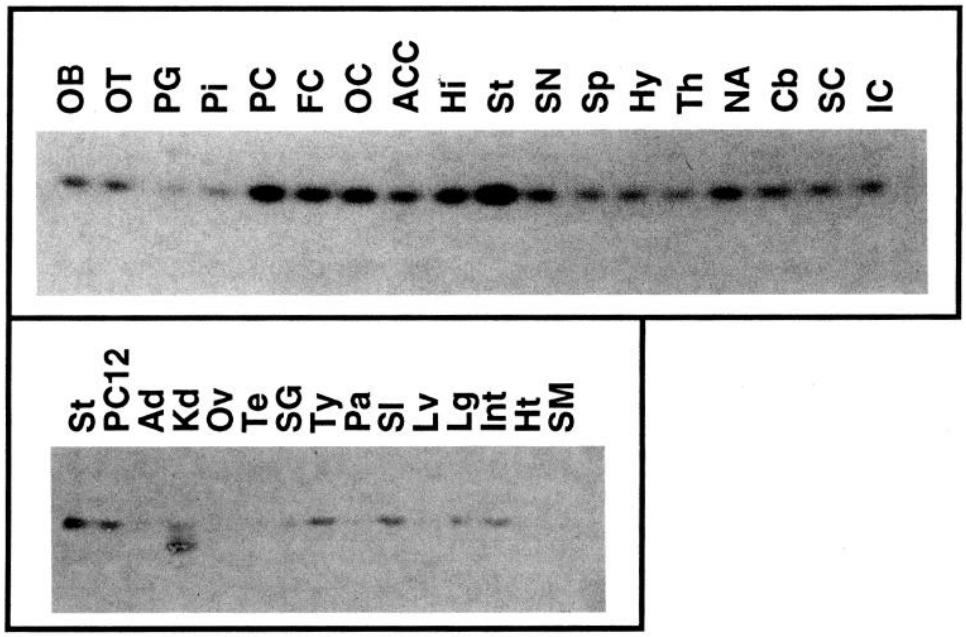

Figure 10. Immunoblot analysis of the distribution of PP1α in rat brain and peripheral tissues. *ACC*, anterior cingulate cortex; *Ad*, adrenal gland; *Cb*, cerebellum; *FC*, frontal cortex; *Hi*, hippocampus; *Ht*, heart; *Hy*, hypothalamus; *IC*, inferior colliculus; *Int*, intestine; *Kd*, kidney; *Lg*, lung; *Lv*, liver; *NA*, nucleus accumbens; *OB*, olfactory bulb; *OC*, occipital cortex; *OT*, olfactory tubercle; *Ov*, ovary; *Pa*, pancreas; *PC*, prefrontal cortex; *PC12*, rat pheochromocytoma immortalized cell line; *PG*, pineal gland; *Pi*, pituitary; *SC*, superior colliculus; *SG*, salivary gland; *Sl*, spleen; *SM*, skeletal muscle; *SN*, substantia nigra; *Sp*, septum; *St*, striatum; *Te*, testis; *Th*, thalamus; *Ty*, thymus.

pression of PP1α and PP1γ1, as determined in this study, with the previously described distribution of DARPP-32 and inhibitor 1 (Hemmings et al., 1992).

Lesion experiments

Following unilateral kainic acid lesion of the striatum, the expression of PP1α and PP1γ1 was investigated by immunoblotting of both ipsilateral and contralateral striatum and substantia nigra. Figure 13 shows a series of typical results and Table 2 summarizes all data. The expression of both PP1α and PP1γ1 was significantly reduced in the lesioned striatum, the former by 33% and the latter by 44% (Table 2). DARPP-32, known to be enriched in the medium-sized spiny neurons of the striatum, was reduced by 53% in the same samples. In contrast, in the lesioned substantia nigra DARPP-32 levels were substantially reduced but not those of PP1α and PP1γ1. As expected, GFAP expression was dramatically increased following kainate lesion, whereas actin and tubulin expression remained relatively unchanged (Fig. 13, Table 2), both in the striatum and in the substantia nigra.

Discussion

Identification of neuronal protein phosphatase 1 isoforms

The cDNA sequence of 1404 nt for rat PP1α is shown in Figure 1. It includes an additional 35 nucleotides of 5′-noncoding sequence compared to the sequence reported by Sasaki et al. (1990), and extends all the way to the poly(A) tail. The deduced amino acid sequence of PP1α is identical to those known from other mammalian species, including rabbit (Bai et al., 1988; Cohen, 1988) and human (Barker et al., 1990; E. F. da Cruz e Silva

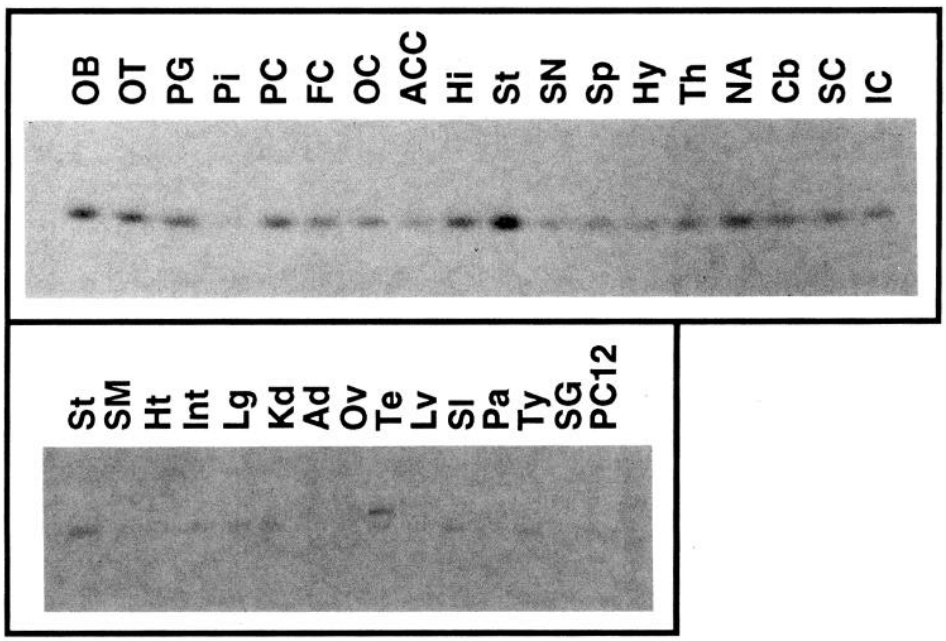

Figure 11. Immunoblot analysis of the distribution of PP1γ1 in rat brain and peripheral tissues. See legend to Figure 10 for key to symbols.

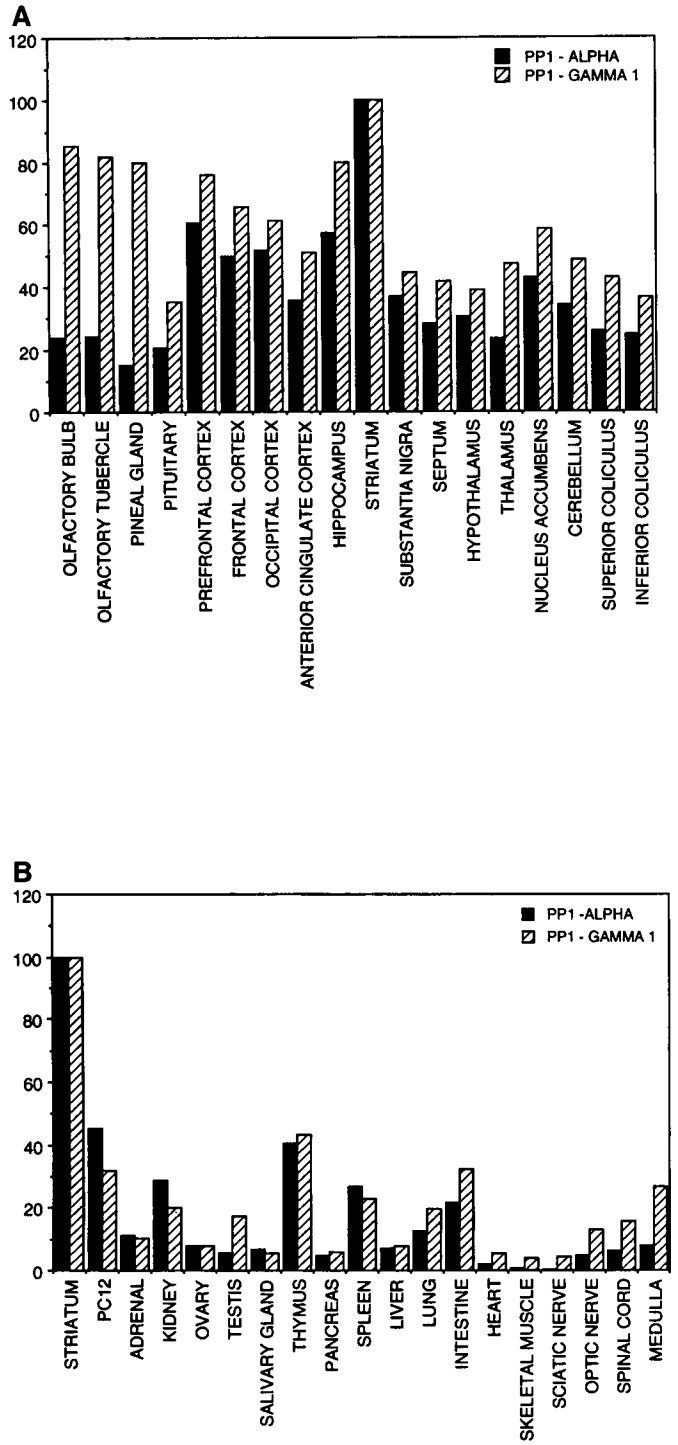

Figure 12. Comparison of the relative abundance of PP1α and PP1γ1 in different rat brain areas (*A*) and peripheral tissues (*B*). Immunoblots were quantitated on a phosphoimager and the results are expressed in relation to the striatum (percentage of striatum).

Table 1. Regional and tissue distribution of PP1α, PP1γ1, DARPP-32 and inhibitor 1

Tissue/area	PP1α	PP1γ1	DARPP-32	Inhibitor 1
Olfactory bulb	23.7	85.4	2.0	116.0
Olfactory tubercle	24.1	81.8	69.0	98.0
Prefrontal cortex	60.4	76.0	4.0	89.0
Frontal cortex	49.6	65.7	5.0	77.0
Occipital cortex	51.7	61.1	5.0	69.0
A. cingulate cortex	35.9	50.7	—	—
Hippocampus	57.2	79.9	3.0	83.0
Striatum	**100.0**	**100.0**	**100.0**	**100.0**
Substantia nigra	37.1	44.4	68.0	85.0
Septum	28.4	41.8	2.0	68.0
Hypothalamus	30.4	39.1	4.0	52.0
Thalamus	23.3	47.1	10.0	85.0
Nucleus accumbens	42.8	58.6	—	—
Cerebellum	34.3	48.4	11.0	36.0
Superior coliculus	26.0	42.9	2.0	49.0
Inferior coliculus	24.7	36.5	2.0	46.0
Medulla	8.0	27.0	—	—
Spinal cord	6.5	15.8	2.0	18.0
Optic nerve	4.7	13.0	—	—
Sciatic nerve	0.38	4.3	—	—
Pineal gland	15.1	79.9	1.0	52.0
Pituitary	20.5	35.2	ND	13.0
Adrenal	11.1	10.4	ND	7.0
Kidney	29.0	20.2	—	—
Ovary	7.8	8.0	—	—
Testis	5.5	17.2	—	—
Salivary gland	6.6	5.5	—	—
Thymus	40.8	43.4	—	—
Pancreas	4.9	6.0	—	—
Spleen	26.7	22.9	—	—
Liver	7.1	7.9	—	—
Lung	12.8	19.9	—	—
Intestine	21.7	32.3	—	—
Heart	1.9	5.4	—	—
Skeletal muscle	0.95	3.8	—	—
PC12	45.4	32.1	—	—

PP1α- and PP1γ1-immunolabeled bands were quantitated on a phosphoimager and are expressed as a percentage of the amount detected in striatum, normalized to an equal amount of protein. ND, not detectable; —, not examined. Data for DARPP-32 and inhibitor 1 were taken from Hemmings et al. (1992), and are shown for comparison.

and P. Greengard, unpublished observations). The cDNA sequence of 2668 nt for rat PP1β is shown in Figure 3. The deduced amino acid sequence of rat PP1β is identical to the mouse dis2m2 sequence (Ohkura et al., 1989). Sasaki et al. (1990) identified a rat PP1γ isoform identical to the one described here (this isoform is referred to as PP1γ1 to differentiate it from the alternatively spliced PP1γ2 isoform that is thought to be expressed only in the testis). Figure 2 comprises 1508 nt of cDNA sequence derived from PP1γ1 that encodes the C-terminal 113 amino acids of the protein. It would appear that the libraries used either did not contain cDNA clones encoding the 5′ portion of PP1γ1, or that such clones were relatively rare. The deduced partial amino acid sequence is identical to both the rat sequence reported by Sasaki et al. (1990) and the human sequence. Indeed, the full length human and rat sequences differ only at a single residue, Ile-4 in rat (and mouse) being replaced by Leu in the human sequence (E. F. da Cruz e Silva and P. Greengard, unpublished observations). The work described here identified PP1α and PP1γ1 in the rat striatum but failed to show the occurrence of either PP1β or any other isoforms by low stringency screening of a rat cDNA library. However, PP1β clones were isolated from a rat cortex cDNA library.

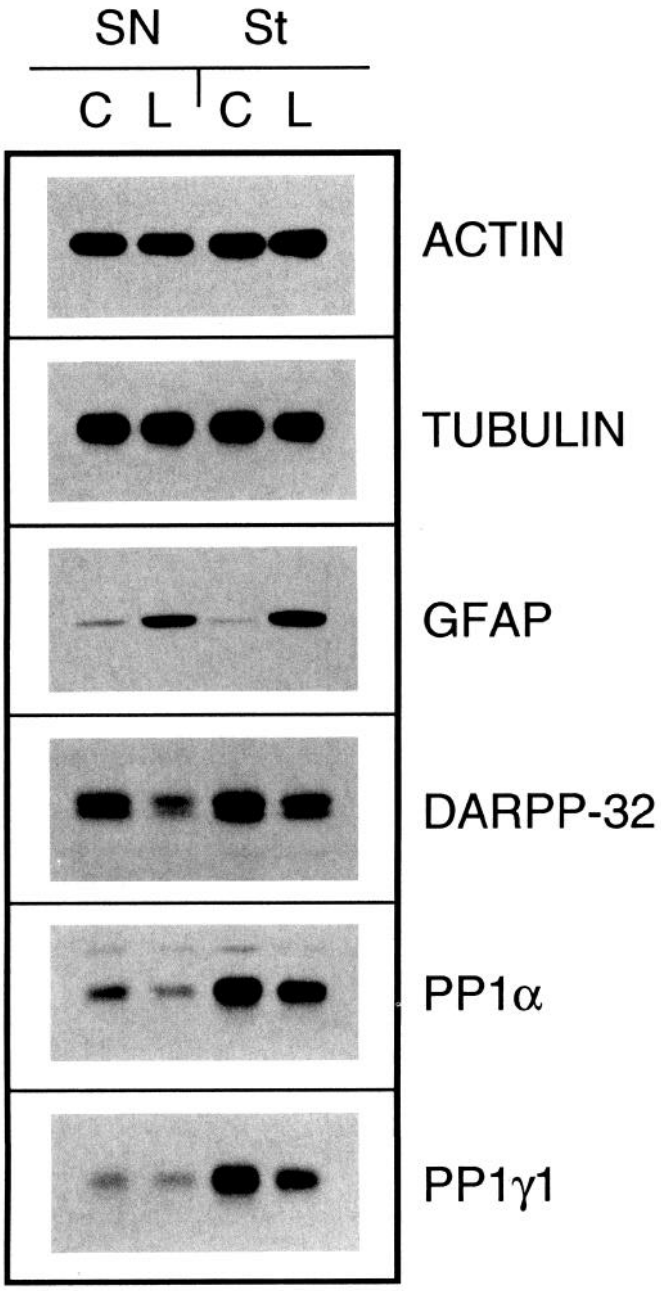

Figure 13. Effect of kainic acid lesion of the striatum on the level of different PP1 isoforms and marker proteins (*C*, control side; *L*, lesioned side). *SN*, substantia nigra; *St*, striatum.

Differential expression of neuronal isoforms of PP1

The distribution of PP1α, PP1β, and PP1γ1 mRNAs in rat brain was investigated by *in situ* hybridization. The results indicate that the three known PP1 genes are widely expressed throughout the rat brain. Broad cortical distribution of mRNA for all three isoforms was observed, but the hippocampus and cerebellum showed particularly strong hybridization for all three probes (Figs. 6, 7). None of the PP1 isoforms is uniquely expressed in the striatum, but there is a somewhat lower level of PP1β signal from this area, in contrast to PP1α and PP1γ1 which were relatively more abundant (Fig. 5). Interestingly, PP1γ1 appears to be uniquely expressed in the islands of Calleja (Fig. 5) and, in addition, it seems to be relatively more abundant than the other isoforms in the pineal gland (Fig. 7). Of the three PP1 isoforms, only PP1β and PP1γ1 show significant expression in the brainstem (Fig. 8).

Table 2. Effect of kainate lesion on the levels of PP1α and PP1γ1

Protein	Striatum	Substantia nigra
Actin	101.2 ± 11.1	97.8 ± 3.6
Tubulin	96.7 ± 4.8	97.4 ± 8.9
GFAP	969.1 ± 366.9	402.0 ± 145.5
DARPP-32	46.6 ± 15.4	46.7 ± 14.9
PP1α	66.9 ± 11.3	82.2 ± 3.4
PP1γ1	56.4 ± 13.3	89.1 ± 7.0

Immunolabeled bands were quantitated on a phosphoimager and results are expressed as percentage of values in the nonlesioned contralateral side. Equal amounts of total protein were loaded in each lane.

The isoform-specific antibodies described here allowed an investigation of the distribution of the individual PP1 isoforms to be carried out by immunoblotting. In general, PP1α and PP1γ1 were found throughout all brain regions and peripheral tissues examined (Fig. 12, Table 1). However, both are relatively more abundant in neuronal tissues. Ironically, skeletal muscle was found to contain relatively modest amounts of PP1, although it is the normal source for protein purification (it is possible that PP1β may be the main isoform expressed in skeletal muscle). PP1α and PP1γ1 are also present in cultured cells including rat pheochromocytoma PC12 cells (Figs. 10, 11) and other immortalized cell lines, including monkey kidney COS cells, mouse neuroblastoma NS20Y cells and human glioma Hs683 (data not shown). However, the highest concentrations of PP1α and PP1γ1 were measured in the striatum (Figs. 10–12, Table 1). Immunoblotting of guinea pig striatum, frontal cortex, and cerebellum yielded results virtually identical to those obtained for rat (not shown).

A comparative analysis of the results obtained by *in situ* hybridization and immunoblotting reveals some superficial discrepancies. The strongest hybridization signals were obtained in the hippocampus and cerebellum, whereas the strongest immunolabeling was observed in striatal homogenates. There are three possible explanations for these results, since the two techniques measure different parameters (mRNA by *in situ* hybridization and protein by immunoblotting). It is possible that the protein and its corresponding mRNA may be localized to different subcellular loci. The relative intensities may also reflect differences in posttranslational regulation of PP1 mRNAs in the various brain areas. Alternatively, the results may be explained by the relative coarseness of the tissue dissection technique (used for immunoblotting), compared to the *in situ* analysis; the neuropil surrounding the highly labeled cell layers in the hippocampus and cerebellum (Figs. 6–8) is virtually devoid of any signal. Thus, the unlabeled tissue would be expected to reduce the signal obtained from crude dissection of these structures.

Lesion experiments. The effects of unilateral lesions of the rat striatum using kainic acid were assessed by immunoblotting of contralateral and ipsilateral striatum and substantia nigra. The levels of actin and tubulin remained relatively constant. In contrast, the levels of GFAP increased dramatically following kainic acid lesion, presumably due to the resulting gliosis. The loss of striatal neurons and their terminals was confirmed by measuring

the levels of DARPP-32. A dramatic decrease in DARPP-32 was observed both in the striatum and in the substantia nigra. We found a substantial and reproducible decrease in the levels of both PP1α and PP1γ1 in the lesioned striatum. However, in the substantia nigra, the decrease for both isoforms, although reproducible, was quite small. Strong immunoreactivity for PP1α and PP1γ1 has been observed in dendritic spines, but only weak immunoreactivity in axons and nerve terminals, at the ultrastructural level (Ouimet et al., in press). The observed distribution at the ultrastructural level could account for the much greater decrease observed in the striatum than in the substantia nigra following striatal lesions.

In a previous study it was found that PP1 activity was not affected by kainic acid lesion of the striatum (Nairn et al., 1988). Those results can be reconciled with the present results if we bear in mind the following points. A substantial proportion of brain PP1 is known to exist in an inactive form. There is also some evidence that it exists in different tissues with different specific activities. At present, no data are available comparing the abundance of different PP1 isoforms and activity in different cell types, for example neuronal cells versus glia. Thus, the immunoblot results presented here are a more reliable measure of phosphatase levels.

The present lesion studies indicate that PP1α and PP1γ1 are expressed within the medium size spiny neurons that project to the substantia nigra, the majority of which are also known to contain DARPP-32 and inhibitor 1. If colocalized within individual neurons, PP1 isoforms may be segregated into subcellular compartments or macromolecular complexes allowing specific regulation by DARPP-32 or inhibitor 1, or fostering interaction with sequestered substrates. Further studies will be required to determine which isoforms of PP1 are colocalized with DARPP-32 and which with inhibitor 1.

Appendix

Abbreviations for figures

ac	anterior commissure
Acb	nucleus accumbens
AMY	amygdaloid complex
AO	anterior olfactory nucleus
CA	CA fields of Ammon's horn
cc	corpus callosum
Cg	cingulate cortex
CG	central gray
CPu	caudate putamen
DB	diagonal band of Broca
DG	dentate gyrus
DR	dorsal raphe
Fr	frontal cortex
Gr	granular layer of the cerebellum
ICj	islands of Calleja
LC	locus coeruleus
LD	laterodorsal thalamic nucleus
MHb	medial habenula
P	pineal gland
Par	parietal cortex
Pn	pontine nuclei
PO	primary olfactory cortex (piriform cortex)
STh	subthalamic nucleus
Tu	olfactory tubercle
Tz	nucleus of the trapezoid body
VM	ventromedial thalamic nucleus
VP	ventroposterior thalamic nucleus
5	trigeminal nuclei

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