Diversity in the Regulatory B-Subunits of Protein Phosphatase 2A: Identification of a Novel Isoform Highly Expressed in Brain[†,‡]

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ABSTRACT: The physiological role of type 2A protein phosphatases (PP2A) is dependent upon the association of the catalytic subunit with a variety of regulatory subunits. In order to understand the function of PP2A, we have undertaken purification of the holoenzymes and molecular cloning of the regulatory subunits. Two trimeric forms containing distinct B-subunits, $PP2A_0$ and $PP2A_1$, have been purified from rabbit skeletal muscle. The B-subunits associated with $PP2A_0$ and $PP2A_1$ migrated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with slightly different mobility, ~52.5 and ~51.5 kDa, respectively and showed distinct immunological properties. The B′ form of B-subunit associated with $PP2A_0$ was recognized by antibodies against the B-subunit present in bovine heart PP2A but not by antibodies specific to the B subunit isoforms of rabbit $PP2A_1$. Cloning of cDNAs encoding the B subunit of $PP2A_1$ resulted in the isolation of a cDNA highly homologous to, but distinct from, the Bα subunit isoform. The deduced amino acid sequence of this novel isoform, which was designated Bγ, encoded a protein which was 81% and 87% identical to the Bα and Bβ isoforms, respectively. Northern blot analysis indicated that the Bγ isoform is highly expressed in rabbit brain as a transcript of 3.9 kb. Analysis of B-subunit expression by Western blot indicated a general parallel with the message levels. In conclusion, our data reveal even greater complexity of PP2A trimeric holoenzymes due to the identification of a novel B regulatory subunit isoform of $PP2A_1$ and a distinct B′ subunit associated with $PP2A_0$.

Reversible phosphorylation of proteins on serine/threonine and tyrosine residues plays a key role in many cellular functions. Serine/threonine phosphatases have been implicated in the regulation of metabolism, receptor and ion channel function, transcription, RNA splicing, cell growth, and transformation (Cohen, 1989; Shenolikar & Nairn, 1991; Walter & Mumby, 1993; DePaoli-Roach et al., 1994). The serine/threonine phosphatases have been classified as types 1, 2A, 2B, and 2C, based on their preferences toward phosphoprotein substrates, sensitivity to inhibitors, and requirements for Ca^{2+} or Mg^{2+} (Cohen, 1989). Most of these enzymes display broad substrate specificity *in vitro*. However, it has been postulated that *in vivo* their activity is more tightly regulated due to association with specific regulatory/targeting proteins (Hubbard & Cohen, 1993). For example, protein phosphatase type 1 associates with G subunit (R_{GL}), inhibitor-2, M subunits, or nuclear inhibitors, NIPP-1, that direct this enzyme to glycogen particles/sarcoplasmic reticulum, cytoplasm, myofibrils, and nucleus, respectively (Stralfors et al., 1985; Tang et al., 1991; Bollen & Stalmans, 1992; Chisholm et al., 1988; Beullens et al., 1992).

The protein phosphatase 2A (PP2A)[1] represents a substantial portion of the serine/threonine phosphatase activity in the cell and has been isolated as complexes containing either two or three polypeptides (Tung et al., 1985; Mumby et al., 1987; Waelkens et al., 1987; Usui et al., 1988; Chen et al., 1989). All purified holoenzymes contained a 36–38 kDa catalytic subunit (C2) associated with a 61–65 kDa regulatory subunit (A subunit). A third, regulatory subunit is present in PP2A trimeric forms. Two such regulatory B-subunits[2] have been characterized, a 52 kDa[3] protein (B subunit) (Healy et al., 1991; Mayer et al., 1991; Pallas et al., 1992) and a 72 kDa protein (Hendrix et al., 1993a), associated with forms of phosphatases termed $PP2A_1$ and polycation-stimulated protein phosphatase M, respectively. Another subunit with an apparent molecular mass of 54 kDa[3] (the B′ subunit) has been reported to be present in a form of phosphatase termed $PP2A_0$ (Tung et al., 1985). However, isolation of this latter form of enzyme has since eluded several laboratories (Mumby et al., 1987; Waelkens et al., 1987; Usui et al., 1988; Chen et al., 1989). The bovine heart PP2A was originally described as containing a B subunit (Mumby et al., 1987), and therefore

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‡ The nucleotide sequences reported in this paper have been submitted to GenBank under Accession Numbers U09354 and U09355.

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[1] Abbreviations: PP2A, protein phosphatase type 2A; C2, catalytic subunit of PP2A; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] The term "B-subunit" is used as a generic descriptor of the third subunit of PP2A; specific polypeptides are designated B, B′, and 72 kDa subunits.

[3] The molecular weights of the B and B′ subunits have usually been reported to be 54 000–55 000 based primarily on the position of the molecular weight marker glutamic dehydrogenase, whose deduced molecular mass is 55 kDa. However, careful calibration of this marker indicates that on SDS–PAGE it actually migrates as a polypeptide of 52–53 kDa. Our determination of the size of the B and B′ subunits is based on a standard curve that takes into account the anomalous migration of glutamic dehydrogenase.

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it appeared to belong to the $PP2A_1$ family.

In vitro studies have indicated that the regulatory subunits may alter the activity and specificity of C2 (Imaoka et al., 1983; Mumby et al., 1987; Usui et al., 1988; Chen et al., 1990; Agostinis et al., 1992). Some insight into the potential role of the B-subunits *in vivo* derives from studies of *Saccharomyces cerevisiae* and *Drosophila* mutants deficient in regulatory subunits. We have previously described that mutation or disruption of the yeast *CDC55* gene (Healy et al., 1991), which encodes a protein highly homologous to the mammalian B subunits, causes defective cytokinesis, cell septation, and/or separation. Also, *Drosophila* mutants deficient in a homologue of the B subunit have been described and characterized. One mutant, termed *aar* (*abnormal anaphase resolution*), shows mitotic defects in neuroblasts, and affected flies die at late pupal or early adult stage (Mayer-Jaekel et al., 1993). Another mutant, termed *twins*, displays overgrowth of the wing imaginal disc (Uemura et al., 1993).

Studies of certain animal viruses also point towards a role for the regulatory subunits in directing C2 to physiological substrates. Some viral proteins associate with PP2A and appear to subvert the function of this enzyme. These proteins include polyomavirus middle T and both polyomavirus and SV40 small t antigens that associate with the C2-A dimer (Pallas et al., 1990; Walter et al., 1990). This replacement of a native phosphatase regulatory subunit by viral proteins could play a role in the viral transformation presumably by stimulation of the MAP kinase pathway (Sontag et al., 1993). Recently, another complex between adenovirus E4orf4 protein and a trimeric form of PP2A has been described (Kleinberger & Shenk, 1993). In this case, E4orf4 appears to complex with PP2A through binding to the B subunit.

In this paper, we report a rapid purification procedure for two trimeric forms of PP2A from rabbit skeletal muscle and the molecular cloning of a novel regulatory subunit, termed Bγ. The highest level of expression of the mRNA encoding this subunit is in rabbit brain. Peptide sequencing and immunoblot analysis of B-subunits associated with both $PP2A_0$ and $PP2A_1$ indicate that the subunit complexed with $PP2A_0$ is distinct from the phosphatase regulatory subunits so far described and that the previously isolated bovine heart form (Mumby et al., 1987) belongs to the $PP2A_0$ family.

EXPERIMENTAL PROCEDURES

Materials. DEAE-cellulose (DE-52), DEAE-Sepharose-CL 6B, poly(L-lysine)–agarose, and ω-aminohexyl-Sepharose 4B were obtained from Sigma and Pharmacia LKB. Thiophosphorylase *a*–Sepharose was prepared by coupling [^{35}S]-phosphorylase *a* to CNBr-activated Sepharose 4B (Pharmacia) according to manufacturer's specifications. Protease inhibitors were purchased from Boehringer Mannheim. Reagents for SDS–PAGE were from Bio-Rad. Restriction enzymes and M13 vectors were from Life Technologies, Inc. GeneScribe-Z vector, pTZ18U, and random hexamer primed DNA labeling kit were obtained from United States Biochemicals. Rabbit brain cDNA library constructed in λgt10 was from Clontech Laboratories, Palo Alto, CA. Radionucleotides were purchased from New England Nuclear and ICN. Recombinant inhibitor-2 was purified as described by Park et al. (1994). Bovine heart PP2A was prepared as described by Mumby et al. (1987). Phosphorylase *b* and phosphorylase kinase were purified from rabbit skeletal muscle as previously reported (DePaoli-Roach, 1984). Thiophosphorylase *a* was prepared essentially by the same procedure as used for [^{32}P]phosphorylase *a* (DePaoli-Roach, 1984) except that [γ-S]ATP was used as the phosphate donor and a trace amount of [γ-^{35}S]ATP was included.

Protein Purification. Freshly prepared rabbit skeletal muscle (2.5 kg) was minced in a meat grinder and homogenized using a Waring blender in 3 volumes of 2 mM EDTA, 2 mM EGTA, pH 7.0, 0.1% 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mM tosyl-L-phenylalanine chloromethyl ketone (TPCK), 2 mM benzamidine hydrochloride, and 2 μg/mL each of leupeptin, antipain, and pepstatin A. The homogenate was centrifuged at 14000*g* for 40 min; the supernatant (6.9 L) was filtered through Miracloth (Calbiochem) and acidified to pH 5.9 with 1 M acetic acid. After being stirred for 15 min, the extract was centrifuged at 14000*g* for 30 min and adjusted to pH 7.2 with 1 M Tris base. One liter of settled DEAE-cellulose (DE-52), previously equilibrated with 20 mM Tris-HCl, pH 7.3, 0.1 mM EDTA, 0.2% 2-mercaptoethanol and protease inhibitors (buffer A), was added to the neutralized extract; the slurry was stirred for 1.5 h and filtered through a Buchner funnel. The DE-52 was washed with 8 L of buffer A containing 50 mM NaCl, and PP2A was batch-eluted with buffer A containing 300 mM NaCl. One 250 mL and five 500 mL fractions were collected. The first three 500 mL fractions which contained 75% of the PP2A activity were combined and fractionated with ultrapure ammonium sulfate. Protein precipitated between 30% and 50% saturation was recovered. The pellet was dissolved in 80 mL of buffer A containing 10% glycerol (buffer B), dialyzed against buffer B, and centrifuged at 80000*g* for 1 h. The resulting supernatant was loaded onto a DEAE-Sepharose-CL 6B column (44.5 × 3.8 cm), and the column was washed with 1 L of buffer B containing 50 mM NaCl at a flow rate of 50 mL/h. The PP2A activity was eluted with a linear gradient of NaCl from 50 to 400 mM, and 10 mL fractions were collected. Two peaks of phosphatase activity were eluted, the first at ~170 mM NaCl and the second at ~250 mM NaCl (Figure 1). Fractions 195–208 from the first peak ($PP2A_0$) and fractions 211–230 from the second peak ($PP2A_1$) of phosphatase activity were pooled, diluted 1:1 with buffer B, and loaded separately on a poly(L-lysine)–agarose column (10 × 1.7 cm). The column was washed with 200 mL of buffer B containing 150 mM NaCl, and PP2A was eluted with a linear gradient of NaCl from 150 to 600 mM. $PP2A_0$ and $PP2A_1$ eluted from poly(L-lysine)–agarose at ~270 and ~370 mM NaCl, respectively. Fractions containing $PP2A_0$ were dialyzed against buffer B containing 100 mM NaCl and loaded onto an ω-aminohexyl-Sepharose 4B column (13 × 1.7 cm). The column was washed with 200 mM NaCl in buffer B, and $PP2A_0$ eluted with a linear gradient of NaCl from 200 mM to 1 M. The peak of $PP2A_0$ activity eluted at ~600 mM NaCl. Chromatography on thiophosphorylase *a*–Sepharose was applied as a final purification step for both $PP2A_0$ and $PP2A_1$. $PP2A_0$ was loaded in buffer B, the column (15.5 × 1.8 cm) washed with buffer B, and the enzyme eluted with a step of 50 mM NaCl in the same buffer. $PP2A_1$ was also loaded in buffer B, the column washed with the same buffer containing 50 mM NaCl, and the enzyme eluted with a linear gradient of NaCl from 50 to 600 mM. Two partially overlapping peaks of phosphatase activity were detected, both eluting at concentrations below 100 mM NaCl. Fractions were adjusted to 30% glycerol and stored at −20 °C. Phosphatase activity was measured at 2 mg/mL phosphorylase *a* (0.9 mol of phosphate/mol of protein, specific radioactivity 3000–4000 cpm/pmol) in the presence of 5 mM caffeine, 5 μg/mL protamine, and 0.1 μM inhibitor-2, and otherwise as

previously described (DePaoli-Roach, 1984). Protein concentration was measured according to the Bradford method (1976) using bovine serum albumin as a standard.

Determination of Amino Acid Sequences. The subunits of $PP2A_0$ and $PP2A_1$ were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970) and transferred to nitrocellulose (Burnette, 1981). Proteins were visualized by staining with 0.1% Ponceau S in 5% acetic acid and bands corresponding to PP2A subunits excised and digested *in situ* with trypsin (Aebersold et al., 1987). The resulting soluble tryptic peptides were separated on microbore HPLC (Healy et al., 1991) and subjected to automated Edman degradation on a Porton Instrument Model 2090 Integrated Micro-Sequencing System (Tarzana, CA) equipped with an on-line HPLC for detection of PTH derivatives.

Molecular Cloning of Bγ cDNA. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer Model 380A. The following degenerate oligonucleotides based on amino acid sequences of peptides obtained from the B subunit of $PP2A_1$ were used: A, 5′-CA(C/T)GA(G/A)CCNGA-(G/A)TT(C/T)GA(C/T)TA-3′, coding, and B, 5′-TA(G/A)TC(G/A)AA(C/T)TCNGG(C/T)TCGTG-3′, noncoding sequences, corresponding to amino acids HEPEFDY; C, 5′-TTCGA(G/A)GA(G/A)CCNGG(G/A)GA(T/C)CC-3′, coding, and D, 5′-GG(A/C)TC(T/C)TCNGG(T/C)TC(T/C)TC(A/G)AA-3′, noncoding sequences, corresponding to amino acids FEEPEDP; E, 5′-GG(C/G)GAGTACAA(C/T)GT(C/G)TA-3′, coding sequence, corresponding to amino acids GEYNVY; and F, 5′-GG(T/C)TTGAT(G/A)TC(G/C)ACGATGTTGAA-3′, noncoding sequence, corresponding to amino acids FNIVDIKP. For some residues in some oligonucleotides, the most frequently used codon(s) in rabbit was (were) chosen. Oligonucleotides were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and duplicate filters containing 1.6×10^5 independent recombinants from a rabbit skeletal muscle random primed cDNA library constructed in λgt11 (Zhang et al., 1989) were screened with a mixture containing either oligonucleotides A, D, and E or oligonucleotides B, C, and F. Prehybridization was carried out at 55 °C in a solution containing 10 × Denhardt's [Ficoll, poly(vinylpyrrolidone) and gelatin at 0.2% w/v each], 6 × SSPE (1 × SSPE: 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4), 0.05% sodium pyrophosphate, and 0.1 mg/mL yeast RNA. Hybridization was performed overnight at 42 °C in the same solution with 4×10^6 cpm/mL radiolabeled oligonucleotides. Filters were washed twice at room temperature for 10 min in 6 × SSC (1 × SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0), 0.05% sodium pyrophosphate, and 0.1% SDS, followed by 20 min at 42 °C, and subjected to autoradiography using X-OMAT AR (Kodak) film with Du Pont Quanta III intensifying screens at −80 °C. Positive clones were plaque-purified by three rounds of screening, the recombinant phage DNA was isolated (Sambrook et al., 1989), and the cDNA inserts were subcloned into the *Eco*RI site of pTZ18U (GeneScribeZ) and M13mp18 for restriction enzyme analysis and sequencing, respectively. Sequencing was carried out by the dideoxynucleotide chain termination method (Sanger et al., 1977) on single-stranded templates with vector-specific and cDNA-specific oligonucleotide primers. Of the four positive cDNA clones analyzed by sequencing, clone RSM7–1 (755 bp) showed high homology to the Bα-subunit cDNA isolated from rabbit skeletal muscle (Healy et al., 1991). Therefore, the rabbit skeletal muscle library (1.7×10^5 recombinants) was rescreened with RSM7–1 cDNA labeled with [α-^{32}P]dCTP by the random-primed method (Feinberg & Vogelstein, 1983) and with a 5′-end-labeled 17-mer oligonucleotide (5′-AGCTCAAGGACCT-GTCC-3′), corresponding to positions 596–612 in the cDNA. The cDNA and the oligonucleotide were used at 68 and 42 °C, respectively, as described above. Two positive clones were isolated, RSM20–1 (807 bp) and RSM20–5 (583 bp), that extended the sequence towards the 3′-end by 222 nucleotides. The next round of screening of 1.9×10^5 recombinants was carried out with ^{32}P-labeled RSM20–5 cDNA and a 20-mer oligonucleotide, 5′-GTGCGACAAGCACTCTCCAAGC-3′, corresponding to positions 954–973 in the Bγ cDNA. Two more clones were isolated, RSM22–2 (940 bp) and RSM29–1 (279 bp), but both overlapped with already available sequences. Therefore, we screened a rabbit brain cDNA library (Clontech; 2.5×10^5 recombinants) with the RSM20–5 cDNA and the 20-mer oligonucleotide as above. Four positive clones were isolated and three of them (RBR4–1, 2.3 kb; RBR4–2, 2.7 kb; and RBR9–1, 3.5 kb) analyzed by sequencing. These clones covered the missing 3′-end of the cDNA but contained sequences unrelated to the muscle clones at their 5′-ends. In order to obtain the 3′-region of the muscle cDNA, the rabbit skeletal muscle cDNA library (4.6×10^5 recombinants) was rescreened with the 907 bp *Pst*I–*Sma*I fragment of the RBR4–1 clone, corresponding to nucleotides 626–1533 in the Bγ cDNA, and a 30-mer oligonucleotide (5′-GTGCACGTCG-GAGTTGACTTTGTCCTGGAA-3′), corresponding to positions 1495–1524 in the Bγ cDNA. Five positive clones were isolated, and two of them, which hybridized to both probes, were sequenced. These cDNA clones (RSM42–1A/B, 1225 bp) were identical and extended the 3′-end sequence.

Sequence analysis was carried out with PCGENE and GCG software packages (Devereux et al., 1984) utilizing the FASTN/FASTP and the FASTA/TFASTA programs, respectively (Pearson & Lipman, 1988).

Northern Blot Analysis. Total cellular RNA was prepared from tissues of male New Zealand rabbits by the guanidinium isothiocyanate–acid phenol method of Chomczynski and Sacchi (1987). RNA samples were separated by 1% agarose/formaldehyde gel electrophoresis and transferred to nitrocellulose in 10 × SSC (Thomas, 1980). A 455 bp fragment, nucleotides 301–755 of the Bγ cDNA clone RSM7–1, and a 348 bp fragment, corresponding to nucleotides 534–881 of the Bα cDNA, were labeled as described above and used as probes. Prehybridization (55 °C, 3 h) and hybridization (2×10^6 cmp/mL, 65 °C, overnight) were carried out in the same solutions as above. Filters were washed twice at room temperature for 10 min in 2 × SSC, 0.05% sodium pyrophosphate, and 0.1% SDS, followed by 20 min wash at 68 °C, and autoradiographed by exposing at −80 °C with intensifying screens.

Preparation of Peptide-Specific Antisera and Immunoblotting. Synthetic peptides corresponding to amino acids 14–26 of Bα isoform, 1–13 of Bβ isoform, and 1–19 of Bγ isoform were coupled to keyhole limpet hemocyanin and used to raise antibodies in rabbits (Bα and Bβ; Kamibayashi et al., 1994) and guinea pigs (Bγ; Tang et al., 1991). MAP peptides (Tam, 1988) corresponding to amino acids 314–324 of the human Aα and 327–337 of Aβ were used directly to prepare antibodies in rabbits. Anti-Cdc55 protein antibodies were obtained from Dr. J. Pringle (Healy et al., 1991). Antiserum E005 was generated against B-subunit derived from PP2A isolated from bovine heart (Kamibayashi et al., 1994). Tissues and cells were disrupted with a Polytron in 50 mM Tris-HCl, pH 7.8, 2 mM EDTA, 2 mM EGTA, 0.1% 2-mercaptoethanol, and a cocktail of protease inhibitors as described for the

Table 1: Purification of Rabbit Skeletal Muscle Protein Phosphatase 2A$_1$ and 2A$_0$

	total protein (mg)	activity (+inhibitor-2 + protamine) (nmol/min)	specific activity (nmol min^{-1} mg^{-1})	purification (x-fold)	yield (%)
		Protein Phosphatase 2A$_1$			
muscle extract	128000	62300	0.49	1	100
pH 5.9 supernatant	121000	44300	0.37	0.8	71
DEAE-cellulose (DE-52)	6200	27800	4.5	9	45
30–50% $(NH_4)_2SO_4$	2100	14900	7.1	14	24
DEAE-Sepharose CL-6B	160	17100	110	220	27
poly(L-lysine)–agarose	21	10000	480	980	16
thiophosphorylase *a*–Sepharose 4B	2.4	9300	3900	7960	15
		Protein Phosphatase 2A$_0$			
DEAE-Sepharose CL-6B	440	4800	11	22	8
poly(L-lysine)–agarose	140	3600	26	53	6
ω-aminohexyl-Sepharose 4B	57	2200	38	78	4
thiophosphorylase *a*–Sepharose 4B	0.8	990	1200	2450	2

purification of PP2A. The extracts were centrifuged at 10000*g* for 20 min, and the soluble fraction was adjusted to a final concentration of 12.5 mM Tris–phosphate, pH 6.8, 0.5% SDS, 10% glycerol, and 1% 2-mercaptoethanol. The samples were subjected to 10% SDS–PAGE and transferred to nitrocellulose (Burnette, 1981). The nitrocellulose filters were blocked overnight at room temperature in PBS (20 mM sodium phosphate, pH 7.4, and 115 mM NaCl) containing 0.1% Tween-20 and 3% nonfat dry milk followed by incubation with antibodies in PBS. Antisera were used at 1:100 to 1:1000 dilution. For the detection of bound antibodies ^{125}I-protein A (0.2 μCi/mL) was used. The autoradiography was carried out at −80 °C with intensifying screens.

RESULTS

Purification of Two Trimeric Forms of PP2A from Rabbit Skeletal Muscle. Two forms of PP2A were purified close to homogeneity from rabbit skeletal muscle by developing a rapid procedure which involved three or four chromatographic steps (Table 1) as compared with previously published protocols which required up to nine steps (Tung et al., 1985; Mumby et al., 1987; Waelkens et al., 1987; Usui et al., 1988). Purification involved one batch-adsorption on DEAE-cellulose, a DEAE-Sepharose-CL 6B, a poly(L-lysine)–agarose, and a thiophosphorylase *a*–Sepharose affinity chromatography. PP2A$_0$ purification required an additional chromatographic step on ω-aminohexyl-Sepharose before the last affinity chromatography since the enzyme did not bind to thiophosphorylase *a*–Sepharose when applied directly after poly(L-lysine)–agarose. Most likely, this was due to the presence of phosphorylase which competed with the ligand.

Two peaks of phosphatase activity eluted at ~170 and ~250 mM NaCl from DEAE-Sepharose-CL 6B (Figure 1), consistent with the elution of PP2A$_0$ and PP2A$_1$ as reported by Tung et al. (1985). No peak of PP2A activity corresponding to the C2–A dimer (PP2A$_2$) eluted after PP2A$_1$ in our studies. Although no data were presented, Tung et al. (1985) indicated that results similar to ours, i.e., absence of a PP2A$_2$ peak, were obtained when a rapid batch-absorption on DEAE-Sepharose of rabbit skeletal muscle extracts was included early in the purification. Two partially overlapping peaks of PP2A$_1$ activity eluted from thiophosphorylase *a*–Sepharose (data not shown) whereas PP2A$_0$ eluted as a single symmetric peak. The two peaks of PP2A$_1$ eluted from the thiophosphorylase–Sepharose column had similar kinetic properties with phosphorylase *a* as a substrate [K_m = 2.7 μM and V_{max} = 2420 nmol min^{-1} (mg of protein)$^{-1}$ versus 2.5 μM and 3180 nmol min^{-1} (mg of protein)$^{-1}$. Based on HPLC profiles of tryptic

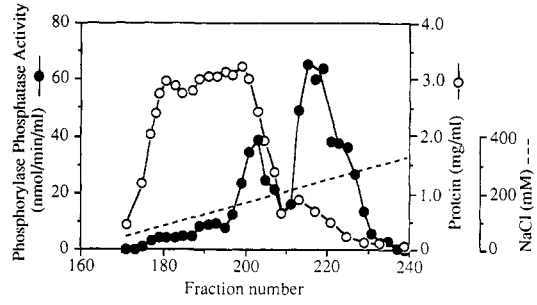


FIGURE 1: DEAE-Sepharose-CL 6B chromatography of rabbit skeletal muscle protein phosphatases 2A. The dialyzed 30–50% ammonium sulfate fraction was applied on the DEAE-Sepharose-CL 6B column (44.5 × 3.8 cm) at a flow rate of 50 mL/h, and 10 mL fractions were collected. The column was washed with 1 L of 50 mM NaCl in buffer B and eluted with a 1.2 L linear gradient from 50 to 400 mM NaCl in buffer B. Phosphorylase phosphatase activity (●) was measured as described under Experimental Procedures in the presence of 5 mM caffeine, 5 μg/mL protamine, and 0.1 μM inhibitor-2. Protein concentration (○) was determined according to the Bradford method. The NaCl concentration is also indicated (- - -).

digests of A, both peaks appeared to contain a similar protein (data not shown). In addition, peptide sequences and Western immunoblotting of samples from either peak indicate the presence of the same Bα subunit. The thiophosphorylase affinity chromatography greatly improved and facilitated the entire preparation. Figure 2 shows the purification achieved at this step for both the 2A$_1$ and 2A$_0$ forms. Although the material loaded onto the column was still quite impure, nearly homogeneous enzyme was eluted. The enzymes consisted of polypeptides corresponding to the A (61.5 kDa) and C2 (36 kDa) subunits and a third protein of ~51.5 kDa[3] and ~52.5 kDa[3], the B and B′ subunits, respectively. Densitometric analyses of Coomassie blue stained gels estimated that the three polypeptides were present at close to a 1:1:1 ratio. The relative sizes of the B-subunits are not consistent with the report of Tung et al. (1985), who determined a lower molecular mass for the B′ subunit (54 kDa) as compared to the B subunit (55 kDa). This discrepancy could be explained either by a difference in the SDS–PAGE system used or by some proteolysis of the proteins. In some samples, an additional faint protein of 97 kDa was detected, which most likely was thiophosphorylase *a* that had leaked from the affinity column. By this procedure (Table 1), 2.5 kg of rabbit muscle yielded 0.8 mg of PP2A$_0$ and 2.4 mg of PP2A$_1$ with specific activities of 1200 and 3900 units/mg, respectively. The activity of the final preparation of both forms was stimulated 2-fold by protamine and was not affected by 0.1 μM inhibitor-2.

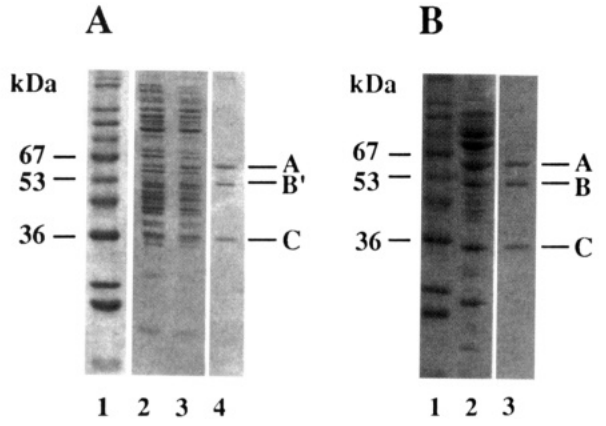


FIGURE 2: SDS–PAGE of $PP2A_0$ and $PP2A_1$ at different stages of purification. Protein samples were electrophoresed on a 10% SDS–polyacrylamide gel and stained with Coomassie Brilliant Blue. Panel A: $PP2A_0$. Lane 1, molecular weight markers; lane 2, phosphatase (5 μg) eluted from ω-aminohexyl-Sepharose 4B; lane 3, flow-through fraction (5 μg) from thiophosphorylase *a*–Sepharose; lane 4, phosphatase (0.2 μg) eluted from thiophosphorylase *a*–Sepharose. Panel B: $PP2A_1$. Lane 1, molecular weight markers; lane 2, phosphatase (4.3 μg) eluted from poly(L-lysine)–agarose; lane 3, phosphatase (0.3 μg) eluted from thiophosphorylase *a*–Sepharose.

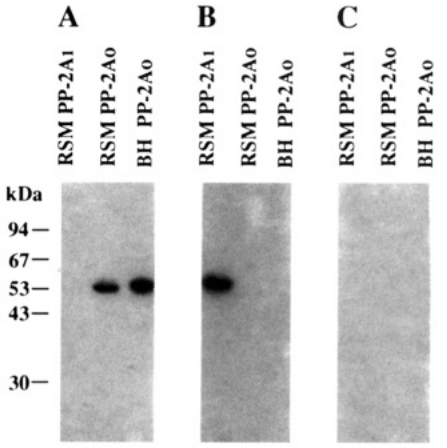


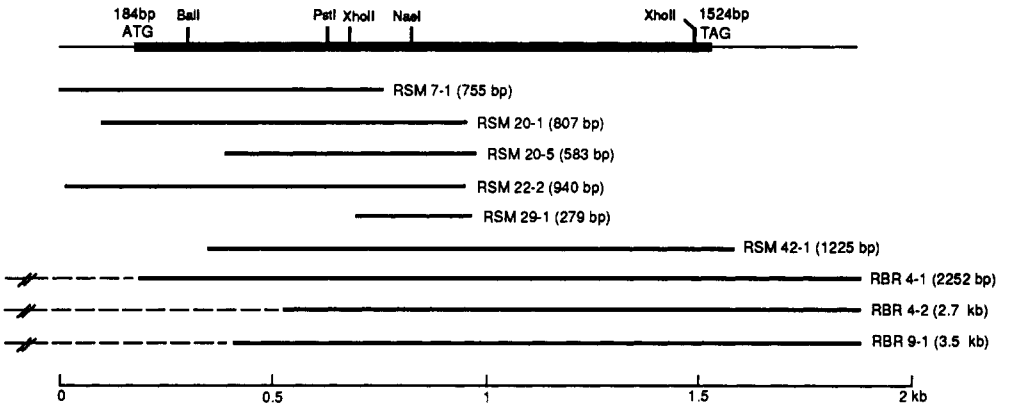
FIGURE 3: Immunoblot analysis of B-subunits. Purified rabbit skeletal muscle (RSM) $PP2A_1$ (lane 1) and $PP2A_0$ (lane 2) and bovine heart (BH) $PP2A_0$ (lane 3) (0.25 μg each) were separated on 10% SDS–PAGE and transferred onto nitrocellulose, and the filters were treated for immunoblot analysis. Panel A was treated with antibodies against the bovine heart PP2A B-subunit (antiserum 1:250 dilution); panel B with Bα specific antibodies (antiserum 1:1000 dilution); panel C with Bβ specific antibodies (antiserum 1:500 dilution).

Characterization of the Subunits Associated with $PP2A_1$ and $PP2A_0$. Two isoforms of the A and C subunits of PP2A have been described (Da Cruz e Silva et al., 1987; Green et al., 1987; Stone et al., 1987; Walter et al., 1989; Hemmings et al., 1990). In an effort to determine the specific isoforms associated with $PP2A_0$ and $PP2A_1$, tryptic peptides derived from the A, C, and B-subunits were sequenced. Sequences of 9 peptides (109 amino acids) were obtained from the B subunit associated with $PP2A_1$ and 7 matched residues in the predicted amino acid sequence of the Bα subunit (Healy et al., 1991). The origin of the two other peptides, GNFEIV and LMIXR, which were not found in the B subunit sequences, is not known at the moment. Sequences of two of the tryptic peptides generated from the A subunit associated with $PP2A_0$ (GPILDNSTLQSEVK and LLAVEACVNIAQLLPQEDLEALV) corresponded to the Aα isoform. These peptides differ in five amino acids from the Aβ isoform (Walter et al., 1989; Hemmings et al., 1990). Sequences of nine peptides that did not correspond to any protein present in the Swiss-Prot 27 data base were obtained for the B′ subunit associated with $PP2A_0$ (C. Csortos, unpublished experiments). Amino acid sequences derived from the C2 subunit in $PP2A_0$ matched conserved regions in the α and β isoforms and did not allow us to identify the isoform present.

Immunological Analyses of Regulatory Subunits Associated with Different PP2A Trimers. To further characterize the B-subunits present in the $PP2A_1$ and $PP2A_0$ holoenzymes, antisera against the B-subunit associated with bovine heart phosphatase, BHPP2A (Mumby et al., 1987), Bα, Bβ, and Bγ peptides, and the yeast Cdc55 protein were used for Western blot analysis. The antiserum against the Cdc55 protein reacted with the B subunit present in rabbit skeletal muscle $PP2A_1$ but not the B′ subunit associated with $PP2A_0$ or the B-subunit of BHPP2A (data not shown). Therefore, Bα and Cdc55, proteins with 53.5% amino acid identity, must share common epitopes. Antiserum E005, generated against the B-subunit associated with the bovine heart PP2A, recognized the B-subunit complexed with BHPP2A and the B′ subunit from rabbit mucle $PP2A_0$, but not the B subunit in $PP2A_1$ (Figure 3, panel A). These results suggest that the bovine heart PP2A purified by Mumby et al. (1987) contains a B′ subunit and therefore represents a $PP2A_0$ form and not a $PP2A_1$ form as previously assumed. Antiserum against the Bα subunit detected only the B subunit associated with rabbit skeletal muscle $PP2A_1$ (Figure 3, panel B) whereas antiserum against the Bβ peptide did not recognize the B-subunit in any of the purified phosphatases (Figure 3, panel C). Furthermore, using antisera raised against a peptide derived from the Bγ-subunit, we were unable to elicit immunoreactivity with any of the polypeptides present in the purified enzymes (data not shown). Antibodies against a human Aα peptide but not against a human Aβ peptide reacted with the A subunit present in both $PP2A_1$ and $PP2A_0$ (data not shown). These results confirm that rabbit skeletal muscle $PP2A_1$ and $PP2A_0$ contain the same isoform of A subunit and most likely C2, but differ in the associated third subunit.

Molecular Cloning of the Bγ Isoform. Initial screening of a rabbit skeletal muscle cDNA library with a mixture of degenerate oligonucleotides based on the sequence of tryptic peptides obtained from the B subunit associated with $PP2A_1$ (Healy et al., 1991) led to the isolation of a 755 bp cDNA clone RSM7–1. This clone contained 183 bp of 5′ noncoding sequence and an open reading frame which was highly homologous to the rabbit Bα cDNA (Healy et al., 1991). Therefore, the protein encoded by the partial cDNA clone, RSM7–1, represented a different isoform of the B subunit and was initially termed Bβ. However, during the course of this work, another isoform of the B-subunit was identified and named Bβ (Mayer et al., 1991). Therefore, for simplicity, the isoform described in this paper is now termed Bγ. Further screening of a total of 5.2×10^5 independent recombinants from the rabbit skeletal muscle cDNA library led to the isolation of four clones (RSM20–1, RSM20–5, RSM22–2, RSM29–1) that did not provide the complete coding sequence, and so another library was used. On the basis of Northern blot analysis, which had shown abundant expression of Bγ mRNA in rabbit brain, a rabbit brain cDNA library was selected for further screening. Four brain library clones were isolated, and three of these contained the 3′-end of the Bγ cDNA. These clones had identical 3′-end sequences, but did not contain a consensus polyadenylation signal motif. Furthermore, the brain library clones contained different sequences at their 5′-ends, none of which matched the sequence of the clones isolated from the rabbit skeletal muscle library. Most likely these differences resulted from concatamerization

A



B

```
   1  GGCGCCCGCCACCCCCCAAGACCTCCCCCGCCGCCCCCGGCCGCGATGCCCACCCCCCGGGGCCCCGGCGGC
  73  CCGCGCCTCTGCTAGGCTGCGGCGGCATGGCCCGCGAGCCCGGCGCGACCTCTGCGGATTGCATCGGTGTGC
 145  GGCGGCGGGGCATGCCCAGGGCACCGGGCACGGCCTTCAATGGGCGAGGACACGGACACGCGGAAAATTAAC
                                          M  G  E  D  T  D  T  R  K  I  N    11
 217  CACAGCTTCCTGCGGGACCACAGCTATGTGACCGAAGCCGATATCATCTCCACGGTCGAGTTCAACCACACC
       H  S  F  L  R  D  H  S  Y  V  T  E  A  D  I  I  S  T  V  E  F  N  H  T    35
 289  GGGGAGCTGCTGGCCACGGGCGACAAGGGCGGCCGGGTCGTCATCTTCCAGCGGGAGCCAGAGAGTAAGAAT
       G  E  L  L  A  T  G  D  K  G  G  R  V  V  I  F  Q  R  E  P  E  S  K  N    59
 361  GCGCCCCACAGCCAGGGCGAGTACGACGTCTACAGCACCTTCCAGAGCCACGAGCCCGAGTTCGACTATCTC
       A  P  H  S  Q  G  E  Y  D  V  Y  S  T  F  Q  S  H  E  P  E  F  D  Y  L    83
 433  AAGAGCCTGGAGATAGAGGAGAAGATCAACAAGATCAAGTGGCTGCCGCAGCAGAACGCTGCCCACTCGCTC
       K  S  L  E  I  E  E  K  I  N  K  I  K  W  L  P  Q  Q  N  A  A  H  S  L   107
 505  CTGTCCACCAACGACAAAACCATCAAGCTATGGAAGATCACCGAGCGAGACAAGAGGCCCGAGGGCTACAAC
       L  S  T  N  D  K  T  I  K  L  W  K  I  T  E  R  D  K  R  P  E  G  Y  N   131
 577  CTGAAGGACGAAGAAGGGAAGCTCAAGGACCTGTCCACGGTGACGTCACTGCAGGTGCCCGTGCTGAAGCCC
       L  K  D  E  E  G  K  L  K  D  L  S  T  V  T  S  L  Q  V  P  V  L  K  P   155
 649  ATGGACCTGATGGTGGAGGTGAGCCCACGGAGGATCTTTGCCAACGGCCACACCTACCACATCAACTCCATC
       M  D  L  M  V  E  V  S  P  R  R  I  F  A  N  G  H  T  Y  H  I  N  S  I   179
 721  TCCGTGAACAGCGACTGCGAGACGTACATGTCGGCCGATGACCTGCGCATCAACCTCTGGCACCTGGCCGTC
       S  V  N  S  D  C  E  T  Y  M  S  A  D  D  L  R  I  N  L  W  H  L  A  V   203
 793  ACCGACAGGAGCTTCAACATCGTGGACATCAAGCCGGCCAACATGGAGGACCTCACCGAGGTGATCACGGCG
       T  D  R  S  F  N  I  V  D  I  K  P  A  N  M  E  D  L  T  E  V  I  T  A   227
 865  TCCGAGTTCCACCCGCACCACTGCAACCTGTTTGTGTACAGCAGCAGCAAGGGCTCCCTGCGGCTCTGCGAC
       S  E  F  H  P  H  H  C  N  L  F  V  Y  S  S  S  K  G  S  L  R  L  C  D   251
 937  ATGCGGGCGGCCGCCCTGTGCGACAAGCACTCCAAGCTCTTTGAGGAGCCCGAGGACCCCAGTAACCGCTCC
       M  R  A  A  A  L  C  D  K  H  S  K  L  F  E  E  P  E  D  P  S  N  R  S   275
1009  TTCTTCTCAGAAATCATCTCATCTGTGTCAGACGTGAAGTTCAGCCACAGCGGCCGGTACATGCTCACGCGG
       F  F  S  E  I  I  S  S  V  S  D  V  K  F  S  H  S  G  R  Y  M  L  T  R   299
1081  GACTACCTCACGGTCAAGGTGTGGGACCTGAACATGGAGGCGAGGCCCATTGAGACCTACCAGGTCCACGAT
       D  Y  L  T  V  K  V  W  D  L  N  M  E  A  R  P  I  E  T  Y  Q  V  H  D   323
1153  TACCTGCGGAGCAAGCTGTGCTCCCTGTATGAGAACGACTGCATTTTCGACAAGTTTGAGTGTGCCTGGAAC
       Y  L  R  S  K  L  C  S  L  Y  E  N  D  C  I  F  D  K  F  E  C  A  W  N   347
1225  GGGAGTGACAGTGTCATCATGACCGGGGCTTACAACAACTTCTTCCGCATGTTCGACCGCAACACCAAGCGA
       G  S  D  S  V  I  M  T  G  A  Y  N  N  F  F  R  M  F  D  R  N  T  K  R   371
1297  GACGTGACCCTGGAGGCCTCGCGGGAGAGCAGCAAGCCCAGGGCCGTGCTGAAGCCGCGGCGCGTGTGCGTG
       D  V  T  L  E  A  S  R  E  S  S  K  P  R  A  V  L  K  P  R  R  V  C  V   395
1369  GGCGGCAAGCGGCGGCGGGACGACATCAGCGTGGACAGCCTGGACTTCACCAAGAAGATCCTGCACACGGCC
       G  G  K  R  R  R  D  D  I  S  V  D  S  L  D  F  T  K  K  I  L  H  T  A   419
1441  TGGCACCCGGCCGAGAACATCATCGCCATCGCCGCCACCAACAACCTGTACATCTTCCAGGACAAAGTCAAC
       W  H  P  A  E  N  I  I  A  I  A  A  T  N  N  L  Y  I  F  Q  D  K  V  N   443
1513  TCCGACGTGCACTAGGGCCCCGGGACGGCCCGGGGGTCGCCCCGCCGCGGTCGGTCGGCGGTCGCT
       S  D  V  H  •                                                           447
```

FIGURE 4: Nucleotide and deduced amino acid sequences of the Bγ isoform of PP2A. Panel A: Diagram of cDNA clones encoding Bγ. The clones were isolated from the rabbit skeletal muscle (RSM) and rabbit brain (RBR) library. Dashed lines at the 5′-end of the rabbit brain clones refer to regions which did not show homology to the muscle clones. A partial restriction map is shown at the top. Panel B: Nucleotide and deduced amino acid sequences of the rabbit muscle Bγ isoform. The amino acid sequence (numbered to the right) is shown below the nucleotide sequence (numbered to the left). The dot represents the stop codon.

of the brain cDNAs since in two clones a polyadenylation sequence was present at the point of divergence.

Figure 4A shows the positions of the Bγ cDNA clones in relation to the open reading frame. Nucleotide and predicted amino acid sequences of the Bγ isoform are shown in Figure 4B. A composite of the muscle clones provides a total of 1578 bp, comprising 183 bp of the 5′ noncoding region, an open reading frame of 1341 bp, encoding a protein of 447 amino acids, and a 3′-noncoding region of 54 bp. The brain clones extended the 3′-untranslated region by 280 bp. The initiator ATG is surrounded by the nucleotide sequence CGGCCT-TCAATGG, which is in reasonable agreement with the consensus motif for initiation of translation in vertebrates (Kozak, 1989). Furthermore, the only other upstream in-frame ATG is followed by a stop codon 36 bp downstream. The calculated M_r of 51 423 is similar to that reported for the rabbit Bα (Healy et al., 1991) and the human Bα and the rat Bβ isoforms (Mayer et al., 1991; Pallas et al., 1992).

Alignment of the amino acid sequences of Bα, Bβ, Bγ, Cdc55, and DPR55–4, the *Drosophila* homolog of the B subunit, is shown in Figure 5. The Bγ isoform is somewhat more similar to Bβ (87% identity) than to Bα (81% identity). The divergence between Bγ and Bα is mainly at the NH_2 termini of the proteins. In contrast, Bγ and Bβ are very similar at their NH_2 termini with only 3 amino acid differences in the first 20 residues and both have a 4 amino acid deletion (VKGA)

```
Bγ       MGEDTDTRKINHSFLR----DHSYVTEADIISTVEFNHTGELLATGDKGGRVVIFQREPESKNAPHSQGEYD   68
Bα       •AGAGGGND•QWC•SQVKGAVDDD•A••••••••••••S•••••••••••••••••Q•Q•N•IQS••R•••N   72
Bβ       •E••I••••••N•••••-------•••A•••••••••••••••••••••••••••••Q••••QV•RR•••N   68
Cdc55    •AQNNFDFKFSQC•G---DKADIV•••••L•TA•••DY••NY••••••••••L•E•SNS------RHC••K   63
DPR55-4  •AG---NGEASWC•SQIKGALDDDY•D•••••C•••••D•••••••••••••••D•A••A•NPRE•••N   69

Bγ       VYSTFQSHEPEFDYLKSLEIEEKINKIKWLPQQNAAHSLLSTNDKTIKLWKITERDKRPEGYNLKDEE----  136
Bα       ••••••••••••••••••••••••••R••••K•••QF••••••••••••S••••••••••E•D----  140
Bβ       ••••••••••••••••••••••••••R••••••••YF••••••••V••••VS•••••••••••••----  136
Cdc55    FLTE••••DA•••••••••••••••E••••RPTQRS•F••••••••••••VY•KNIKLVSQ•NLT•GVTFA  135
DPR55-4  ••••••••••••••••••••••••R••Q•K•PV•F••••••••V••••VS••••SFG•••T•E•N----  137

Bγ       ------------GKLKDLSTVTSLQVPVLKPMDLMVEVSPRRIFANGHTYHINSISVNSDCETYMSADDLRI  196
Bα       ------------•RYR•PT•••T•R•••FR•••••••A••••••••A•••••••••I•••Y•••L•••••••  200
Bβ       ------------•R•R•PA•I•T•R••••R•••••••AT•••V•••A••••••••••••Y••••••••••  196
Cdc55    KKGKPDNHNSRG•SVRAVLSLQ••KL•Q•SQH•KIIAAT•K••YS•A•••••••••L•••Q••FL•••••••  207
DPR55-4  ------------•LIR•PQN••A•R••SV•QIP•L••A••••T•••A••••••••••••Q••FL•••••••  197

Bγ       NLWHLAVTDRSFNIVDIKPANMEDLTEVITASEFHPHHCNLFVYSSSKGSLRLCDMRAAALCDKHSKLFEEP  268
Bα       •••••EI•••••••••••••••E•••••••A••••NS••T••••••••TI•••••••S••••R•••••••••  272
Bβ       •••NFEI•NQ••••••••••••E•••••••A••••••••T••••••••TI•••••••S••••R•T•F••••••  268
Cdc55    •••N•DIP•Q•••••••••T•••E•••••SA••••QE••••M••••••TIK•••••QNS•••NKT•T•••Y  279
DPR55-4  •••••E•VNQ•Y•••••••T•••E•••••••A••••TE••V•••••••TI••••••S•••••R•••Q••••  269

Bγ       EDPSNRSFFSEIISSVSDVKFSHSGRYMLTRDYLTVKVWDLNMEARPIETYQVHDYLRSKLCSLYENDCIFD  340
Bα       ••••••••••••••I••••••••••••M•••••S••I••••••N••V••••••E•••••••••••••••••••  344
Bβ       ••••••••••••••I••••••••••••IM•••••••••••••N••••••••••••••••••••••••••••••  340
Cdc55    L••I•HN••T••T••I••I•••PN•••IAS•••••••I••V••DNK•LK•INI•EQ•KER•SDT••••A•••  351
DPR55-4  •N•T••••••••••I••••L•N•••••IS••••SI•••••H••TK•••••P••E•••A••••••••••••  341

Bγ       KFECAWNGSDSVIMTGAYNNFF----------------------------------------------------  362
Bα       ••••C•••••••V•••S•••••-----------------------------------------------------  366
Bβ       ••••V•••••••••••••••••-----------------------------------------------------  362
Cdc55    •••VNFS•DS•SV•••S•••N•MIYPNVVTSGDNDNGIVKTFDEHNAPNSNSNKNIHNSIQNKDSSSSGNSH  423
DPR55-4  ••••C•••K••S••••S•••••-----------------------------------------------------  363

Bγ       --RMFDRNTKRDVTLEASRESSKPR----------------------AVLKPRRVCVGGKRRRDDISVDSLD  410
Bα       --••••••••••I•••••••NN•••------------------------T•••••K••AS•••KK•E•••••••  414
Bβ       --••••••••••••••••••N•••-------------------------I••••K•••••••K•E•••••••  410
Cdc55    KR•SNG•••GMVGSSNS••S•IAGGEGANSEDSGTEMNEIVLQADKT•FRNK•YGSLAQRSARNKDWG•DI•  495
DPR55-4  ---V••••S•K•••••••DII••K----------------------T•••••K••T••••KK•E••••C••  411

Bγ       FTKKILHTAWHPAENIIAIAATNNLYIFQDKVNSDVH                                    447
Bα       •N••••••••••K•••••V•T•••••••••••                                        447
Bβ       •S••••••••••S•••••V••••••••••••                                         443
Cdc55    •K•NN••FS•••R••S••V••••••F••SAL                                         526
DPR55-4  •N••••••••••E•••••V••••••F•••••F                                        443
```

FIGURE 5: Alignment of amino acid sequences of different B subunits. The Bα sequence is identical in man, rat, and rabbit. Bβ corresponds to the human, and partial rat and rabbit sequences. Cdc55 and DPR55-4 correspond to the *S. cerevisiae* and *Drosophila* homologs of B regulatory subunits. The dots indicate identities and the dashed lines gaps.

as compared with the same region in the Bα protein. The Bγ isoform extends by four amino acids (SDVH) at the COOH terminus as compared to Bβ. At the nucleotide level, the identity between rabbit Bγ and Bα or Bβ is also high, 69% with Bα and 78% with Bβ in the coding region. The identity of Bγ to yeast Cdc55 and *Drosophila* DPR55–4 is also high, 54 and 73%, respectively. If conservative replacements are considered, the homology is 67% and 82%, respectively.

Expression of B Subunit mRNAs and Protein in Rabbit Tissues. To establish whether the mRNA of B subunits displayed tissue-specific expression, Northern analysis was performed with total RNA isolated from several rabbit tissues (Figure 6). The Bγ isoform is encoded by a single transcript of 3.9 kb which is very abundant in brain, barely detectable in skeletal muscle, and undetectable in other tissues examined (Figure 6, panel B). In contrast, the Bα isoform is encoded by transcript(s) of ~2.5 kb present in all tissues examined with the highest level observed in brain and testis and the lowest in liver (Figure 6, panel A). The higher molecular weight band detected in brain samples with the Bα-specific probe most likely represents some degree of cross-hybridization with the Bγ isoform.

To correlate the levels of mRNA with those of the proteins, Western blot analysis was carried out on extracts prepared from bovine and rabbit tissues as well as from bovine smooth muscle and mouse cardiocyte cell lines. Utilizing Bα-specific antibodies, the amount of immunoreactive protein in the tissues examined was found in general to parallel the mRNA levels

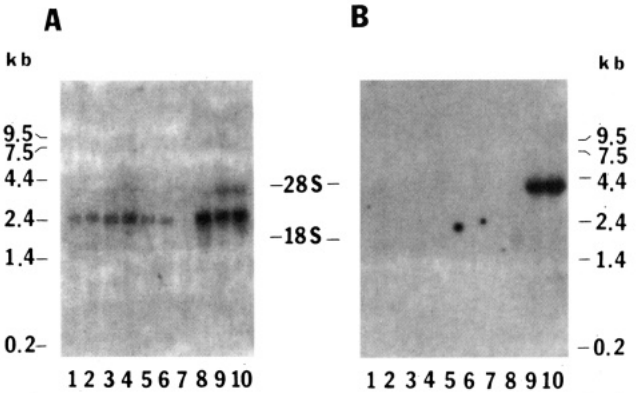


FIGURE 6: Tissue distribution of Bα and Bγ mRNA. Total RNA (20 μg each) from different rabbit tissues was hybridized with Bα (A) and Bγ (B) specific cDNA probes. The Bα probe was a 348 bp fragment (nt 534–881), and the Bγ probe was a 455 bp fragment (nt 301–755). Lane 1, kidney; lane 2, spleen; lane 3, lung; lane 4, heart; lanes 5 and 6, skeletal muscle; lane 7, liver; lane 8, testis; lanes 9 and 10, brain.

(Figure 7, panel A). With antibodies raised against Bβ, high levels of protein were detected in rabbit brain and testis, aorta smooth muscle, and cardiocyte cells, and low levels in kidney, spleen, and lung (Figure 7, panel B). In rabbit skeletal muscle, polypeptides of higher molecular mass, 59 and 93 kDa, which were nonetheless competed by the peptide antigen, were also observed (Figure 7, panel B). Since only a 5′-end-truncated cDNA has been cloned from rabbit skeletal muscle (Mayer

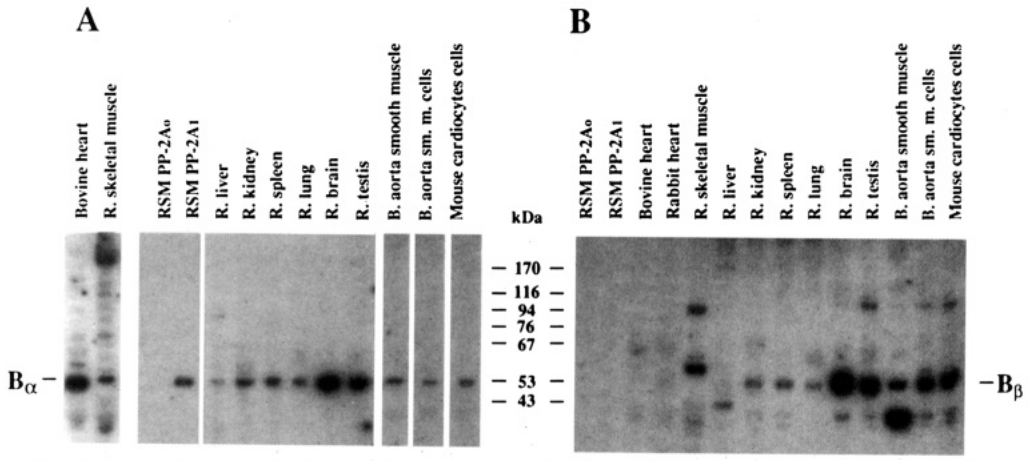


FIGURE 7: Tissue distribution of the α and β isoforms of the B subunit of PP2A$_1$. Soluble protein extracted from the indicated rabbit tissues (40 μg/lane) and cell lines (10 μg/lane) were separated on 7.5% SDS–polyacrylamide gels and immunoblotted with Bα (panel A) and Bβ (panel B) specific antisera as described in the legend of Figure 3. R, rabbit; RSM, rabbit skeletal muscle; B, bovine; sm. m., smooth muscle.

et al., 1991), the existence of alternatively spliced mRNAs, encoding larger polypeptides, cannot be ruled out. The identity of the lower molecular weight species detected in aorta smooth muscle is not clear, and could be due to partial proteolysis. The distribution of the Bβ protein is not consistent with the mRNA distribution reported by Mayer et al. (1991), which indicated a neuronal-specific expression of Bβ mRNA. However, it is possible that the antibody raised against the Bβ peptide might also recognize Bγ protein since in this region there are only three amino acid differences. Nevertheless, these studies indicate tissue-specific expression of B-subunit isoforms.

DISCUSSION

The PP2A protein phosphatase is one of the major serine/threonine protein phosphatase activities present in cells and is implicated in many essential cellular functions. Its subunit structure, a matter of considerable potential importance to understanding its function, has nonetheless proven to be a difficult issue over the years. To begin with, there has been some controversy over the number of subunits present in the enzyme. PP2A has been purified in several laboratories in both dimeric and trimeric forms [see reviews by Cohen (1989) and Shenolikar and Nairn (1991)]. We report here a rapid purification procedure which yielded only trimeric PP2A with no indication of any dimeric form. This result raises the possibility that, in previous preparations of two-subunit PP2A (Tung et al., 1985; Mumby et al., 1987; Waelkens et al., 1987; Usui et al., 1988; Chen et al., 1989), the B-subunit might simply have been lost from trimeric PP2A to generate the dimeric form. The B-subunit could have been dissociated from the trimeric PP2A or could have been subjected to degradation. Results from other studies support this hypothesis. For example, in their purification of a dimeric PP2A from rabbit reticulocytes, Chen et al. (1989) observed that their dimer was generated from a more complex structure, based on estimations of the Stoke's radius. Other studies have also suggested that the B-subunit of PP2A is especially susceptible to degradation by proteases (Pato & Kerc, 1986; Usui et al., 1988; Chen et al., 1989; Kamibayashi et al., 1991). It is therefore possible that the dimeric PP2A forms are artifactually derived from physiologically relevant trimeric species.

The trimeric PP2A also exists in multiple forms. Tung et al. (1985) had originally defined distinct PP2A$_0$ and PP2A$_1$ species, although the isolation of PP2A$_0$ appears not to have been repeated until the present study. We confirm the existence of PP2A$_0$ and now know that it is distinguished by containing a B-subunit, B′, unrelated to the B subunits found in PP2A$_1$. Indeed, the results of this study indicate that the bovine heart PP2A isolated by Mumby et al. (1987) was a PP2A$_0$ holoenzyme and not PP2A$_1$ as originally thought. Another trimeric form of PP2A, designated polycation-stimulated phosphatase M, has been purified from rabbit skeletal muscle (Waelkens et al., 1987). This enzyme consists of C2, A, and a polypeptide of 72 kDa. Cloning of cDNAs encoding the 72 kDa protein suggests the existence of two alternatively spliced forms of 72 and 130 kDa (Hendrix et al., 1993a). With our purification protocol, we did not observe any form of PP2A containing the 72 kDa protein. We cannot explain this negative result and can only surmise that the polycation-stimulated phosphatase M was lost at some step in our procedure. In our purification, we did detect two forms of PP2A$_1$, separable on thiophosphorylase *a*–Sepharose, even though both the A and B subunits appeared to be identical. Heterogeneity of the C2 subunit could explain this observation. Recent studies have described posttranslational modification of C2 by phosphorylation at tyrosine (Chen et al., 1992, 1994) and threonine residues (Guo & Damuni, 1993), as well as carboxymethylation at the COOH-terminal Leu residue (Lee & Stock, 1993; Xie & Clarke 1993, 1994). Thus, it is possible that the heterogeneity observed in our preparations may be due to such modifications.

The heterogeneity of PP2A in general is linked importantly to the existence of different isoforms of its constituent subunits. In the present study, we report the cloning of a new cDNA corresponding to Bγ, a third isoform of the family of B subunits. Bγ is highly homologous, 81% identity, to Bα (Healy et al., 1991) but even more similar, 87% identity, to the Bβ isoform. In the 20 NH_2-terminal amino acids, which is the most divergent region among the B isoforms, Bγ and Bβ differ only by 3 amino acids. Search of the Swiss-Prot data bank with the Bγ amino acid sequence matched several expected proteins, including the mammalian Bα and Bβ, as well as the yeast Cdc55 protein and the *Drosophila* DPR55. In addition, a 378 bp clone HHCPB41 (EST01650; Adams et al., 1992), isolated from a human hippocampus library subtracted with a fibroblast cell line (WI38) cDNA library, was highly homologous to the Bγ sequence from nucleotides 220 to 596. The alignment of the EST01650 and Bγ sequences indicated the insertion of a T at position 290 of the hippocampus-derived clone, but otherwise the sequences were 91% identical. Also,

the predicted amino acid sequence encoded by the clone EST01650, after deletion of the T, would be identical to the rabbit Bγ protein between residues 13 and 137. In this region, there are 28 and 16 amino acid differences between Bγ/Bα and Bγ/Bβ, respectively. This suggests that the clone EST01650 corresponds to the human Bγ isoform. Recently, the EST01650 clone was assigned to human chromosome 4 by analyzing fluorescently labeled PCR products from hybrid cell panels (Durkin et al., 1994). Other evidence for multiple variants of the B subunit came from the work of Pallas et al. (1992), who reported at least six immunoreactive variants of the B-subunit from rat skeletal muscle detected on two-dimensional Western blots. The calculated isoelectric points for rabbit Bα (6.13), Bβ (6.41), and Bγ (6.28) are large enough for separation by isoelectric focusing. To explain six variants, either more B isoforms exist or some of the known ones are subject to posttranslational modifications. Searches of the protein sequences of the B subunit isoforms by the Protein Motifs program indicated multiple potential sites for phosphorylation by both protein kinase C and casein kinase II present in all proteins. In addition, both Bα and Bβ, but not Bγ, appear to have a consensus sequence for phosphorylation by tyrosine kinase.

The mRNA encoding the Bγ isoform is abundant in rabbit brain, and minimally expressed in the other tissues tested. The Bβ isoform mRNA is present at high levels in both rat brain and testis but is undetectable in other tissues (Hatano et al., 1993). In contrast, the Bα isoform mRNA is present in most rabbit (this study) and rat (Hatano et al., 1993) tissues although the highest levels of expression are again seen in brain and testis. A brain-specific role for the B subunit has been suggested in *Drosophila* (Mayer-Jaekel et al., 1993). *Drosophila aar* mutants that are deficient in the B subunit (DPR55) of PP2A display abnormal metaphase–anaphase transition in neuroblasts.

The concept is evolving that the specificity of function of the relatively small number of distinct protein serine/threonine phosphatase catalytic subunits is conferred by a combinatorial association with separate regulatory subunits (DePaoli-Roach et al., 1994; Mayer et al., 1991). Cloning of a third isoform of the B subunit fits with this idea. The findings that the B′ subunit of $PP2A_0$ is unrelated to the B subunit of $PP2A_1$ (C. Csortos, unpublished experiments) and that the 72 kDa subunit of polycation-stimulated phosphatase M is also a distinct protein suggest that the B-subunit of PP2A displays the most variability. Interestingly, it is the B-subunit that is substituted by virally encoded proteins when cells are infected with polyoma or SV40 viruses. A somewhat analogous situation may occur with the cantharidin binding protein. Cantharidin is the toxic constituent of blister beetles, and its binding protein is complexed in rat liver to a Cβ–Aα dimer (Li & Casida, 1992). Besides B-subunit diversity, two isoforms have also been described for the C2 and A subunits of PP2A (Green et al., 1987; Da Cruz e Silva et al., 1987; Stone et al., 1987; Walter et al., 1989; Hemmings et al., 1990). Some of the diversity of PP2A isoforms may be related to tissue-specific expression. For instance, Hatano et al. (1993) found that in rat testis the expression of Bβ mRNA was specific to elongated spermatids, whereas Bα mRNA was expressed equally in all spermatogenic cells. In another study, Hendrix et al. (1993b) observed that in *Xenopus* the Aβ isoform was expressed predominantly in oocytes, but in frog skeletal muscle both Aα and Aβ were present at similar levels. Our results suggest that the Bβ/γ proteins are more tissue-specific whereas the Bα isoform is more ubiquitously expressed. These observations are consistent with the existence of distinct PP2A holoenzyme made up of specific combinations of different subunit isoforms. Simply considering $PP2A_1$, the current count of 2 A, 2 C, and 3 B subunits would provide for up to 12 different trimeric holoenzymes. These could potentially display distinct properties and functions in the cell.

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